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(54) Title: ANGIOTENSIN II_{cAMP}/VASOPRESSIN$_{V2}$ RECEPTORS AND RELATED MOLECULES AND METHODS

(57) Abstract

Disclosed are cDNAs encoding angiotensin II/vasopressin$_{V2}$ (AII/AVP$_{V2}$) receptors, the recombinant proteins expressed from such cDNAs, and antibodies specific for such proteins. The recombinant receptor and receptor analogues are used in methods of screening candidate compounds for their ability to antagonize interaction between AII or AVP and an AII/AVP$_{V2}$ receptor; antagonists are used as therapeutics to treat hypertension. The disclosed cDNAs and receptor protein and receptor protein analogues are used to screen individuals for hypertension or for a propensity toward hypertension.

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ANGIOTENSIN II_{cAMP} / VASOPRESSIN$_{V2}$ RECEPTORS AND RELATED MOLECULES AND METHODS

Background of the Invention

This invention relates to receptors, particularly angiotensin II/ vasopressin receptors.

Angiotensin II (AII) and vasopressin (arginine-vasopressin, AVP) receptors are both G protein-coupled receptors with diverse physiological roles (Crane et al., *J. Biol. Chem.* 257:4959, 1982; Rogers et al., *J. Pharmacol. Exp. Ther.* 236:438 1986; Douglas, *Am. J. Physiol.* 253:F1, 1987; Jard, *Curr. Top. Mem. Transp.* 18:255, 1983; Jard, *Adv. Nephrol.* 16:1, *Physiol. Rev.* 57:313, 1977; Capponi et al., in *Biochemical Regulation of Blood Pressure*, R.L. Soffer, p. 205, John Wiley & Sons, New York, 1981; Smith, *Am. J. Physiol.* 250:F759, 1986). AII receptors respond to the octapeptide hormone and neurotransmitter, angiotensin II, effecting a variety of cell-specific responses including: synthesis and secretion of aldosterone by adrenal glomerulosa cells; vascular smooth muscle and cardiac contractility; stimulation of thirst and salt appetite centers and secretion of vasopressin in the brain; induction of hepatocyte glycogenolysis and gluconeogenesis; induction of the absorption of sodium and water in the intestine; and regulation of renal hemodynamics and tubular transport (Peach, 1977, supra; Capponi et al., 1981, supra; Smith, 1986, supra). Equally diverse, AVP receptors respond to a nonapeptide hormone, arginine-vasopressin, affecting vasoconstriction and vasodilation; positive and negative cardiac chronotropy; regulation of the secretion of corticotropin by the adenohypophysis and increased firing rate of specific neuronol groups in the

brain; induction of hepatocyte glycogenolysis and gluconeogenesis; and increased water reabsorption by collecting ducts and increased solute transport by ascending limb of Henle's loop in the kidney (Jard, 1983, supra; Jard, 1987, supra).

Consistent with this functional diversity, isoreceptors have been described for both AII and AVP based on differing coupling/effector pathways and affinity profiles to various agonist and antagonists. For AII receptors, two non-correlated classifications have been described, each with two subtypes. In kidney, type A is functionally coupled to the cAMP mobilizing effector pathways, and type B is negatively coupled to the adenylate cyclase pathways (Douglas, 1987, supra). In adrenal gland, types 1 and 2 have been described, based on differential anatomical localization of nonpeptide ligand binding (Chiu et al., *Biochem. Biophys. Res. Comm.* 165:196, 1989). For AVP receptors, at least two classes of isoreceptors have been named: type 1 (V1), functionally coupled to calcium mobilizing effector pathways; and type 2 (V2), frequently designated an antidiuretic type, coupled to the adenylate cyclase system and found in kidney (Jard, 1983, supra).

Summary of the Invention

In general, the invention features recombinant angiotensin II_{cAMP}/vasopressin$_{V2}$ (i.e., AII/AVP_{V2}) receptor polypeptide, preferably, including an amino acid sequence substantially identical to the amino acid sequence shown in Fig. 1 (SEQ ID NO: 1). The invention also features a substantially pure polypeptide which is a fragment or analog of an AII/AVP_{V2} receptor and which includes a domain capable of binding angiotensin II (AII) or arginine-vasopressin (AVP) (see below).

In various preferred embodiments, the r c ptor is derived from a mammal, preferably, a human or a rat.

Th invention further features a polypeptid including an AII-binding porti n of an AII/AVP_{V2} receptor, preferably, including amino acids 392 to 399 of Fig. 1 (SEQ ID NO: 1); a polypeptide including an AVP-binding portion of an AII/AVP_{V2} receptor, preferably, including amino acids 342 to 350 of Fig. 1 (SEQ ID NO: 1); and a polypeptide including an extracellular domain of an AII/AVP_{V2} receptor or an immunogenic analog thereof, preferably, including amino acids 30-94, amino acids 151-251, amino acids 338-390, or amino acids 437-481 of Fig. 1 (SEQ ID NO: 1) and, more preferably, including amino acids 193-200 of Fig. 1 (SEQ ID NO: 1), or an immunogenic analog thereof. The polypeptide may be a recombinant polypeptide.

By "AII/AVP_{V2} receptor polypeptide" is meant all or part of a cell surface protein which specifically binds AII and AVP and signals the appropriate AII- and AVP-mediated cascade of biological events (leading, for example, to an increase in intracellular cAMP). By a "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation). A "substantially pure polypeptide" is one which is substantially free of other proteins, carbohydrates and lipids with which it is naturally associated. By a "substantially identical" amino acid sequence is meant an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the biological activity of the receptor. Such equival nt r ceptors can be isolated by extraction from the tissues or cells of

any animal which naturally produce such a receptor or which can be induced to do so, using the methods described below, or their equivalent; or can be isolated by chemical synthesis; or can be isolated by standard techniques of recombinant DNA technology, e.g., by isolation of cDNA or genomic DNA encoding such a receptor. By "derived from" is meant encoded by the genome of that organism and present on the surface of a subset of that organism's cells.

In another related aspect, the invention features purified DNA which encodes a receptor (or fragment or analog thereof) described above. Preferably, the purified DNA is cDNA; is purified DNA which encodes a rat AII/AVP_{V2} receptor; is purified DNA which encodes a human AII/AVP_{V2} receptor; is included in the plasmid pSVL-A1/V9; and is included in the plasmid pMAM-DR-AII/AVP_{V2}.

By "purified DNA" is meant a DNA molecule which encodes an AII/AVP_{V2} receptor (or an appropriate receptor or analog), but which is free of the genes that, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene encoding the AII/AVP_{V2} receptor.

In other related aspects, the invention features vectors which contain such purified DNA and are capable of directing expression of the protein encoded by the DNA in a vector-containing cell; and cells containing such purified DNA (preferably eukaryotic cells, e.g., mammalian cells, e.g., COS 1 cells or C127 cells).

The expression vectors or vector-containing cells of the invention can be used in a method of the invention to produce recombinant AII/AVP_{V2} receptor polypeptide and the receptor fragments and analogues described above. The method involves providing a cell transformed with DNA encoding an AII/AVP_{V2} receptor or a fragment or analog thereof positioned for expression in the cell; culturing

the transformed cell under conditions for expressing the DNA; and isolating the recombinant AII/AVP_{V2} receptor protein. By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding an AII/AVP_{V2} receptor (or a fragment or analog, thereof). Such a DNA molecule is "positioned for expression" meaning that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of the AII/AVP_{V2} receptor protein, or fragment or analog, thereof).

In yet another aspect, the invention features purified antibody which binds preferentially to an AII/AVP_{V2} receptor (or a fragment or analog thereof). By "purified antibody" is meant one which is sufficiently free of other proteins, carbohydrates, and lipids with which it is naturally associated to permit therapeutic administration. Such an antibody "preferentially binds" to an AII/AVP_{V2} receptor (or fragment or analog, thereof), i.e., does not substantially recognize and bind to other antigenically-unrelated molecules.

Preferably, the antibody neutralizes <u>in vivo</u> the protein to which it binds. By "neutralize" is meant to partially or completely block receptor-ligand binding.

The invention further features a method of testing a candidate compound for the ability to inhibit binding of AII or AVP to an AII/AVP_{V2} receptor. The method involves: a) contacting the candidate compound with a recombinant AII/AVP_{V2} receptor (or AII- or AVP-binding fragment or analog), preferably expressed on the surface of a recombinant cell, and with AII or AVP; b) measuring binding of AII or AVP to the receptor (or receptor fragment or analog); and c) identifying antagonist compounds as those which decrease such binding.

Preferred antagonists are those which also reduce the AII- or AVP-mediated increase in the intracellular cAMP concentration of a cell bearing the recombinant receptor or receptor fragment or analog on its surface.

By an "antagonist" is meant a molecule which also inhibits a particular activity, in this case, inhibition of the ability of AII or AVP to bind an AII/AVP_{V2} receptor and, preferably which inhibits the biological events normally resulting from such binding (e.g., an increase in intracellular cAMP concentration).

The antagonists (i.e., the polypeptides or antibodies described above) are used as the active ingredient of therapeutic compositions. In such therapeutic compositions, the active ingredient may be formulated with a physiologically-acceptable carrier or anchored within the membrane of a cell.

The therapeutic compositions are used in a method of treating AII- or AVP-mediated disorders, including increased contraction of blood vessels leading to hypertension. The method involves administering the therapeutic composition to a mammal in a dosage effective to inhibit binding of AII or AVP to an AII/AVP_{V2} receptor.

The proteins of the invention are involved in mediating the effects of angiotensin II and vasopressin (AII and AVP, respectively); cells bearing AII/AVP_{V2} receptors derive (without limitation) from the kidney, the liver, the central nervous system, the heart, and the vasculature. The diverse processes likely regulated by the proteins of the invention include water reabsorption and solute transport in the kidney; chronotropy and inotropy of the heart; stimulation of thirst and salt appetite centers in the brain; induction of the absorption of sodium and water in the intestine; and, of particular interest in the instant invention, modulation of blood vessel contraction. Such proteins are therefore

useful to treat or, alternatively, to develop therapeutics to treat hypertension and, generally, AII- or AVP-mediated disorders of the vascular system (e.g., stroke triggered, at least in part, by hypertension).

Preferred therapeutics include antagonists e.g., peptide fragments, antibodies, or drugs, which block AII or AVP ligand or AII/AVP_{V2} receptor function by interfering with the AII or AVP: receptor interaction.

Because the receptor component may now be produced by recombinant techniques and because candidate antagonists may be screened in vitro, the instant invention provides a simple and rapid approach to the identification of useful therapeutics. Such an approach was previously difficult because of the presence on the surface of AII/AVP_{V2} receptor-bearing cells (e.g., vascular cells) of related receptors. Isolation of the AII/AVP_{V2} receptor gene (as cDNA) allows its expression in a cell type remote from those cells on whose surface the receptor normally resides, effectively providing a system for assaying an AII:receptor or AVP:receptor interaction without interference caused by ligand interaction with related receptors.

Once identified, a peptide- or antibody-based therapeutic may be produced, in large quantity and inexpensively, using recombinant and molecular biological techniques.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Detailed Description

The drawings will first briefly be described.

Drawings

Fig. 1 is the nucleotide sequence and deduced amino acid sequence of the AII/AVP_{V2} receptor (SEQ ID NO: 1).

Fig. 2 is a tabular representation of the effect of AVP on cAMP accumulation is Xenopus laevis oocytes which were microinjected with A1/V9 mRNA.

Fig. 3 is a graphical representation of AII-induced and AVP-induced accumulation of cAMP in Cos 1 cells (A) and Cos A1/V9 cells (B).

Fig. 4 A and B are bar graphs showing the effects of various putative ligands and antagonists on cAMP accumulation is Cos A1/V9 cells.

Fig. 5 is a graphical representation of cAMP accumulation in Cos A1/V9 cells as a function of AII concentration (A) or AVP concentration (B).

Fig. 6 is a tabular representation of the pharmacologic parameters of the AII/AVP_{V2} receptor.

Fig. 7A is a graphical representation of a dissociation analysis of AII binding to Cos A1/V9 cells; Fig. 7B is a Scatchard plot of the results of Fig. 7A.

Fig. 8A is a graphical representation of a saturation analysis of AVP binding to Cos A1/V9 cells; Fig. 8B is a Scatchard plot of the results of Fig. 8A.

Fig. 9 is a graphical representation of a competition binding analysis of various AII and/or AVP agonists or antagonists.

Fig. 10 is a hydropathy analysis of the AII/AVP_{V2} receptor.

Fig. 11 is the putative structure of the AII/AVP_{V2} receptor.

Fig. 12 A and B are graphical representations of (A) AVP-induced or (B) AII-induced cAMP accumulation in cells expressing either wild-type or mutant AII/AVP_{V2} receptors.

Fig. 13 is a tabular representation of the effect of NaCl on AVP-dependent and AII-dependent cAMP accumulation.

Polypeptides According To The Invention

Polypeptides according to the invention include the entire human AII/AVP_{V2} receptor and the entire rat AII/AVP_{V2} receptor (as described in Fig. 1; SEQ ID NO: 1). These polypeptides are used, e.g., to screen for antagonists which disrupt an interaction between AII or AVP and the receptor (see below). Polypeptides of the invention also include any analog or fragment of the human AII/AVP_{V2} receptor or the rat AII/AVP_{V2} receptor capable of interacting with AII or AVP. Such analogues and fragments may also be used to screen for AII/AVP_{V2} receptor antagonists. In addition, that subset of receptor fragments or analogues which bind AII or AVP and are, preferably, soluble (or insoluble and formulated in a lipid vesicle) may be used as antagonists to reduce AII/AVP_{V2} receptor-mediated disorders, e.g., those described herein. The efficacy of a receptor analog or fragment is dependent upon its ability to interact with AII or AVP; such an interaction may be readily assayed using any of a number of standard in vitro binding methods and AII/AVP_{V2} receptor functional assays (e.g., those described below).

Specific receptor analogues of interest include full-length or partial (see below) receptor proteins including an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the receptors' ability to bind AII or AVP (e.g., as assayed below).

Specific receptor fragments of interest include any portions of the AII/AVP_{V2} receptor which are capabl

of interaction with AII or AVP_{V2}. Such a portion preferably includes amino acids 392-399 or 342-350 of Fig. 1 (SEQ ID NO: 1) or an AII or AVP-binding portion (respectively), thereof. Such fragments may be useful as antagonists (as described above).

The extracellular domains (i.e., amino acids 30 to 94; amino acids 151 to 251; amino acids 338 to 390; and amino acids 437 to 481) or fragments thereof (preferably, amino acids 193-200) are also useful as a source of immunogens for producing antibodies, e.g., those which neutralize the activity of the AII/AVP_{V2} receptor in vivo (e.g., by interfering with the interaction between the receptor and AII or AVP).

From the primary amino acid of the AII/AVP_{V2} receptor sequence, the secondary protein structure and, therefore, the extracellular domain regions may be deduced semi-empirically using a hydrophobicity/hydrophilicity calculation such as the Chou-Fasman method (see, e.g., Chou and Fasman, *Ann. Rev. Biochem.* 47:251, 1978). Hydrophilic domains, particularly ones surrounded by hydrophobic stretches (e.g., transmembrane domains) present themselves as strong candidates for extracellular domains. Finally, extracellular domains may be identified experimentally using standard enzymatic digest analysis, e.g., tryptic digest analysis.

Candidate fragments are tested for interaction with AII or AVP by the assays described herein. Such fragments are also tested for their ability to antagonize the interaction between AII or AVP and its endogenous receptor using the assays described herein. Analogues of useful receptor fragments (as described above) may also be produced and tested for efficacy as screening compon nts or antagonists (using th assays described

her in); such analogues are also considered to be us ful in the invention.

There now follows a description of the cloning of an AII/AVP_{V2} receptor-encoding cDNA useful in the invention and a characterization of its ligand binding properties. This example is provided for the purpose of illustrating the invention, and should not be construed as limiting.

Cloning and Characterization of the Rat AII/AVP_{V2}Receptor

The rat AII/AVP_{V2} receptor gene was isolated as follows.

Oligonucleotides were designed based on the complementary mRNA sequence of the rat AVP ligand and the rat AII ligand (Ohkubo et al., *Proc. Natl. Acad. Sci. USA* 80:2196, 1983; Ivell and Richter, *Proc. Natl. Acad. Sci. USA* 81:2006, 1984). These oligonucleotides, of 24 and 26 bp in length, respectively were obtained from Research Genetics (Huntsville, AL) and were of sequence:

5′ AAA GGG GTG GAT GTA TAC GCG GTC 3′ (i.e., the AII oligonucleotide; SEQ ID NO:2); and

5′ TCC TCT TGG GCA GTT CTG GAA GTA GCA 3′ (i.e., the AVP oligonucleotide; SEQ ID NO:3).

The oligonucleotide probe was ^{32}P end-labelled as described in Sambrook et al. (*Molecular Cloning : A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY, 1989) and used to screen an adult rat kidney cDNA library obtained from Clontech (Palo Alto, CA). Hybridization was carried out using 10^6 cpm/ml probe and the hybridization buffer: 6X SSPE [i.e., 1M NaCl, 60mM NaH_2PO_4 (pH 7.4), 6mM EDTA (pH 7.4)], 100 ug/ml denatured calf thymus DNA, 0.1% sodium pyrophosphate, 1% sodium dodecyl sulfate (SDS), and 200 ug/ml polyadenylic acid. Filters were washed 3 times in 2X SSPE, 0.1% pyrophosphate, 0.1% SDS at 40°C; each wash was carried out for 15 minutes.

From 10^6 recombinant clones, six cDNA clones were independently isolated using the AII oligonucleotide probe and nine cDNA clones were independently isolated using the AVP oligonucleotide probe. The longest of the isolated cDNA clones (and those larger than the AII or AVP mRNAs) were termed: A1 (i.e., putative AII receptor cDNA #1) and V9 (i.e., putative vasopressin receptor cDNA #9). These clones were shown to be identical cDNA clones by size (i.e., ≈2.25 kb) and restriction mapping analysis. Hybridization experiments (carried out by standard techniques) confirmed that both the A1 and V9 cDNAs hybridized to both the AII and AVP oligonucleotide probes and to each other, even under stringent hybridization conditions.

Expression of the A1/V9 cDNA was investigated by RNA blot analysis (as described in Herrera and Ruiz-Opazo, *Science* 249:1023, 1990). Using the 2.25 kb A1/V9 cDNA as a hybridization probe and stringent hybridization conditions, two-size classes of mRNA were detected in rat kidney (i.e., ≈2.4 and 2.5 kb). The 2.5 kb mRNA species was also detected in the following rat tissues (in order of abundance): kidney > brain > lung vasculature > heart > skeletal muscle > aorta > adrenal gland.

Functionality of the A1/V9 receptor cDNA was also investigated; specifically, the A1/V9 cDNA was expressed in either *Xenopus laevis* oocytes or mammalian cells, and activation or inhibition of the adenylate cyclase system by AVP and AII was determined as follows.

The 2.25 kb A1/V9 cDNA was subcloned, in both orientations, into the EcoRI site of the transcription vector, pSP73 (Promega Corp, Madison, WI), and the clones were arbitrarily designated, A1/V9(+) and A1/V9(-). *In vitro* transcribed RNAs, A1/V9(+) RNA and A1/V9(-) RNA, respectively, were obtained using SP6 RNA polymerase-direct d transcription of the A1/V9 cDNA subclones and

the manufacturer's specifications (Promega Corp., Madison, WI). RNA was isolated by th m thod of Herrera and Ruiz-Opazo (Science 249:1023,1990) and microinjected into Xenopus laevis. Xenopus laevis oocyte expression experiments were carried out essentially as described in Colman (in *Transcription and Translation*, pp. 271-302 eds. Hames and Higgins, IRL PRESS, Oxford, 1984) with the following specifications: full length A1/V9(-) and A1/V9(+) RNA concentrations were ascertained by RNA blot analysis and densitometric quantitation of the autoradiographic signal (as carried out by standard techniques); 75 ng of each RNA was injected in 50 nl water; prior to microinjection, each oocyte was checked for the complete removal of its vitelline membrane, and vitelline membrane remnants were mechanically removed. Oocyte membranes were isolated as described in Colman (1984, supra), and the adenylate cyclase assay was conducted as described in Murayama and Ui (*J. Biol. Chem.* 259:761, 1984).

Membranes (20 ug) from the two groups of microinjected Xenopus oocytes, A1/V9(-) and A1/V9(+), were assayed for adenylate cyclase activity under three different experimental conditions: addition of 0.1 μM AVP (Sigma, St. Louis, MO), addition of 10 mM sodium fluoride (NaF), and incubation medium only (control). Cyclic AMP generated during incubation, in pmols/20 minute incubation/mg protein, was measured by a sensitive radiommunoassay method (i.e., cAMP [^{125}I] Assay System, AMERSHAM Corp; Arlington Heights, Inc.). Values shown in Fig. 2 represent the means $\pm$ S.D. of three experiments; each point being an average of duplicate determinations per experiment.

Only one orientation of the A1/V9 cDNA-derived in vitro transcripts, i.e., A1/V9(+) RNA, showed AVP-induced cAMP accumulation. As shown in Fig. 2, the A1/V9(+) RNA

microinjected oocyte membranes elicited a 2-fold increase in cAMP accumulation upon addition of 0.1 μM AVP. The A1/V9(-) RNA microinjected oocyte membranes did not show any increase in cAMP levels as compared to the basal levels but did have a 3-fold increase in cAMP accumulation upon the addition of 10 mM sodium fluoride (equal to that observed for the A1/V9(+) RNA-microinjected oocyte membranes), demonstrating that the adenylate cyclase system in the A1/V9(-) RNA-microinjected oocyte membranes were functionally active. From this experiment, it was concluded that A1/V9(+) contained the 2.25 kb A1/V9 cDNA inserted in the sense orientation.

The A1/V9 cDNA was then expressed by insertion (in the sense orientation) into the pSVL expression vector (PHARMACIA, Piscataway, NJ) and transfection into Cos 1 tissue culture cells. Specifically, the 2.25 kb A1/V9 cDNA was excised by digestion with XhoI and EcoRV and subcloned directionally (5′ to 3′) into the XhoI-SmaI sites of the pSVL expression vector, to create plasmid pSVL-A1/V9. pSVL-A1/V9 was co-transfected into Cos 1 cells (i.e., Green monkey kidney cells, ATCC Accession No. CRL 1650, American Type Culture Collection, Rockville, MD) in a 20:1 ratio with the plasmid, pSV2Neo, a plasmid which confers neomycin resistance. A mixed population of stable neomycin resistant transfectants, termed Cos A1/V9 cells, were selected with 500 ug/ml G418 antibiotic (effective concentration = 250 ug/ml as per manufacturer's specifications GIBCO; Grand Island NY). Cells were maintained in G418 for the duration of the expression studies. Control mock-transfected cells were developed in parallel. Cos 1 cells were cotransfected with an unrelated cDNA-pSVL expression plasmid and pSV2neo in a 20:1 ratio, and an identical selection in G418 was performed.

Transfectants were analyzed for the presence of the A1/V9 cDNA sequences. Southern blot analysis of genomic DNA obtained from Cos A1/V9 cells showed multiple copies of high molecular weight integrated (>8kb) and non-integrated (7kb) pSVL-A1/V9 sequences. As expected, at stringent hybridization conditions, no A1/V9-specific sequences were noted in the control mock-transfected Cos 1 cells. In addition, digestion of Cos A1/V9 cellular DNA with EcoRI released the expected 2.25 kb fragment including the uninterrupted A1/V9 cDNA sequence. Moreover, complete A1/V9-specific polymerase chain reaction-amplified products were detected only in Cos A1/V9 cells; no product was detected in the mock-transfected Cos 1 cells or untransfected Cos 1 cells. Finally, the presence of A1/V9 mRNA was detected in the poly(A)$^+$ RNA isolated from Cos A1/V9 cells but not in the RNA isolated from the control mock-transfected cells. Isolation of genomic DNA and Southern blot analysis were performed as described in Herrera and Ruiz-Opazo (*Science* 249:1023, 1990). Poly(A)$^+$ RNA blot analysis was also performed as described in Herrera and Ruiz-Opazo (1990, supra). PCR amplification was carried out as described in *PCR Protocols: A Guide to Methods and Applications* (eds, Innis et al., 1990) using A1/V9-specific primers.

Transient and permanent Cos A1/V9 transfectants were assayed for receptor function. Because of a higher and more consistent level of expression, dissection of receptor function was subsequently carried out in the permanent Cos A1/V9 transfectants only. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) in 48-multiwell dishes. Cells were then pre-incubated in DMEM containing 20 mM Hepes, pH 7.4, and 100 μM IBMX (3-isobutyl-1-methylxanthine; Sigma, St. Louis, MO) for 20 minutes at room temperature; followed by incubation for 2 minutes in PSS buffer (118 mM NaCl, 4.7 mM KCl, 3 mM

$CaCl_2$, 1.2 mM $MgSO_4$, 1.2mM KH_2PO_4, 0.5 mM EDTA, 10 mM glucose, 20 mM Hepes, pH 7.4) with or without (control) the test hormone at specified doses and with or without antagonist depending on experimental design. The level of cAMP was determined by radioimmunoassay according to manufacture's specifications (specifically cAMP [^{125}I] Assay System, AMERSHAM). Reactions were terminated by the addition of 2 volumes of 100% ethanol to the cells. To standardize the data with respect to variations of cAMP levels among independent but concordant groups of experiments, results were expressed as the percent stimulation of cAMP accumulation with respect to the zero time point.

Control (i.e., untransfected and mock-transfected) Cos 1 cells (Fig. 3A) and Cos A1/V9 cells (Fig. 3B) were exposed to AII (△) and AVP (▲). A basal control (●), i.e. no hormone added, was included for comparison. cAMP accumulation was measured from 30 seconds to 5 minutes. Each time point was performed in duplicate; the percent range of variation was 0.06-6% with a mean percent variation of 2%.

The Cos A1/V9 transfectants (Fig. 3B) showed 60-70% AII-induced and 90-100% AVP-induced stimulation of cAMP accumulation over the control untransfected and mock-transfected Cos 1 cells. The cAMP levels increased briskly reaching plateau levels at about 1 minute and the responses to AVP were consistently greater than the responses to AII by a difference of approximately 30%. Results from un-transfected and mock-transfected cells were identical; both exhibited minimal, if any, cAMP accumulation (Fig. 3A).

Specificity for both AII and AVP was ascertained through development of an agonist/antagonist response profile in Cos A1/V9 cells.

The % stimulation of cAMP accumulation was measured in response to the peptide ligands: AVP, AII, angiotensin I (AI), angiotensin III (AIII), bradykinin, and endothelin 1 (Et1), all at 0.1 μM. Specificity for AII stimulation was tested by concurrent incubation of AII at 1 nM and antagonists at 100 nM. Likewise, specificity for AVP stimulation was tested by concurrent incubation of AVP at 1 nM and antagonists at 100 nM. Experiments were carried out as described above. Results are shown in Fig. 4A and Fig. 4B.

The Cos A1/V9 cells did not respond to the peptide hormones, endothelin-1 or bradykinin. The rank order for % stimulation of cAMP accumulation by the different angiotensin peptides was as follows: AII ≥ AI ≥ AIII. In addition, exposing Cos A1/V9 cells concurrently with AII and a hundred-fold excess of the classical AII antagonists, [Sar^1, Ala^8]-AII; [Sar^1, Ile^8]-AII; and [Sar^1, Thr^8]-AII (Pals et al., *Circ. Res.* 29:673, 1971; Khosla et al., *J. Med Chem.* 15:792, 1972; Munoz-Ramirez et al., *Res. Comm. Chem. Path. Pharmacol.* 13:649, 1976); or AVP (V1/V2) antagonist, [$d(CH2)_5$, D-$I13^2$, Ile^4]-AVP (Manning et al., *J. Med. Chem.* 27:423, 1984) efficiently blocked the AII- and AVP-induced cAMP accumulation, respectively (Fig. 4A). When done in experimental conditions as in Fig. 4A, i.e., with 1 nM ^{3}H-AVP, complete displacement was noted with 100 nM of V1/V2 antagonist. The AII antagonists did not block the AVP-induced cAMP accumulation, nor did the V1/V2 antagonist block the AII-induced cAMP accumulation. This is consistent with the existence of two independent binding domains for AII and AVP.

In the representative experiment shown in Fig. 4B, the % stimulation of cAMP accumulation was measured in response to AVP, AII, and AVP + AII (all at 0.1 uM). Experiments were carried out as described above; at least

three separate experiments were performed per set with each point performed in duplicate. AII and AVP combined did not elicit additive stimulation of cAMP accumulation, consistent with a single putative receptor responding to the two ligands. In addition, the AII/AVP receptor was found not to be functionally coupled to a Ca^{2+} mobilizing effector pathway, as determined by measurement of unidirectional Ca^{2+} efflux by the method of Brown et al. (*J. Biol. Chem.* 259:7554, 1984).

To further analyze the dual hormone response profile of the AII/AVP_{V2} receptor, dose-response curves were generated for both AII and AVP. cAMP levels (expressed in fmol/2 minutes/10^4 cells) were measured in response to varying concentrations of AII (Fig. 5A) and AVP (Fig. 5B). Each point is the mean ± range of variation (I, not indicated when bar size graphically undetectable) from at least three separate experiments with each point performed in duplicate. The range of percent variation was 0.1-5.2% (Fig. 5A) and 0.1-2.5% (Fig. 5B) with mean percent variations of 1.7% and 0.9%, respectively. Results shown in Fig. 5 were used to calculate an EC_{50} value for both AII and AVP of 0.1 nM (Fig. 6). These EC_{50} values were significantly low and confirmed the specificity of response to both AII and AVP. The dual hormone response profile of the AII/AVP receptor was further indicated by the k_D values for ^{125}I-AII binding (i.e. K_H = 0.05 nM and K_L = 6.4 nM; Fig. 6) and for ^3H-AVP binding (K_L = 5.9 nM; Fig. 6). Validity of these values for specificity for both AII and AVP were borne out by the fact that they were comparible to, if not better than, previously published studies assessing AII and AVP receptors in isolated membranes or intact cells (Crane et al., *J. Biol. Chem.* 257:4959, 1982; Rogers et al., *J. Pharmacol. Exp. Ther.* 236:438, 1986; Jard, 1983, supra; Jard, 1987, supra).

To further characterize the pharmacologic properties of the AII/AVP receptor, competition and saturation binding studies were performed using either ^{125}I-AII or ^{3}H-AVP and intact Cos A1/V9 cells. Binding experiments were carried out as described by Rogers et al. (Rogers et al., 1986, supra) with the following specifications: for ^{125}I-AII binding, each assay point consisted of 10^6 cells cultured in P-35 dishes; binding assays were done in 1 ml of binding buffer (Rogers et al., 1986, supra) containing the appropriate concentration of ligand; cells with bound ^{125}I-AII were removed with 1 ml of 0.25 N NaOH, 0.25% SDS; for ^{3}H-AVP binding, each assay point consisted of 3 X 10^6 cells cultured in P-60 dishes; binding assays were done in 2 ml of binding buffer with the appropriate concentration of ligand; and cells with bound ^{3}H-AVP were removed with 2 ml of 10 mM Tris/HCl, pH 7.4, 10 mM EDTA, 3% Triton X-100. All incubations were performed at 37°C for 20 minutes. Specific binding was determined as the difference between the total radioactivity bound to cells and the radioactivity bound to blanks containing 1 μM AII or 10 μM AVP. The specific activities of the radiolabeled peptides were 2000 Ci/mmol [^{125}I-AII] and 50 Ci/mmol [^{3}H-AVP]. No specific binding of ^{125}I-AII or ^{3}H-AVP was detected in control untransfected or mock-transfected Cos 1 cells.

Fig. 7A shows the dissociation analysis of ^{125}I-AII specific binding performed on intact Cos A1/V9 cells. Each point represents the mean ± range of variation (I) of three separate experiments with each point performed in duplicate. The percent variation was 0.1-6%; mean percent variation was 3.1%. Fig 7B shows a Scatchard plot (LIGAND Program, McPherson) of the results of Fig. 7A. Two affinity sites are depicted. Affinity values and corresponding B_{max} values are presented in Fig. 6.

Results of the ^{125}I-AII displacement curve were analyzed for both AII affinity sites (i.e., the low affinity site, K_L, and the high affinity site, K_H). B_{max} values are shown in fmols/10^6 cells.

Fig. 8A illustrates the saturation analysis of ^{3}H-AVP specific binding to intact Cos A1/V9 cells. Each point represents the mean ± range of variation (I) of three separate experiments with each point performed in duplicate. The percent variation was 0.8-13%; mean percent variation was 5%. Fig. 8B shows a Scatchard plot of the results of Fig. 8A. Affinity and B_{max} values are presented in Fig. 6.

Scatchard analysis of ^{125}I-AII competition binding and ^{3}H-AVP saturation binding (Figs. 6, 7, and 8) revealed the presence of a single class of binding site for AVP [i.e., of low affinity (K_L = 5.9 nM) and high capacity (B_{max} = 6.0 fmols/10^6 cells)] and two classes of binding sites for AII [i.e., one of high affinity (K_H = 0.05nM) and low-capacity (B_{max} = 0.38 fmols/10^6 cells) and one of low affinity (K_L = 6.4 nM) and high-capacity (B_{max} = 6.7 fmols/10^6 cells)]. The total number of binding sites for AII (B_{max} = 7.08 fmols/10^6 cells) and AVP (B_{max} = 6 fmols/10^6 cells) were similar, consistent with the hypothesis that the same receptor recognizes both AII and AVP as ligands in Cos A1/V9 cells.

In addition, the similarity of the K_d value for the high-affinity AII-binding site (0.05 nM), and the AII EC_{50} value for the stimulation of adenylate cyclase (0.1 nM) (Fig. 6) suggested that agonist occupancy of these high affinity binding sites might account for the measured activation of the adenylate cyclase system. The observation of two distinct classes of AII binding sites on the AII/AVP receptor was consistent with the characterization of high and low affinity sites of AII receptors in the liver (Crane et al, *J. Biol. Chem*

257:4959, 1982) and heart (Rogers et al., 1986, supra). Due to the low specific activity of [^{3}H]-AVP, (50 Ci/mmol), only receptor binding sites with affinities in the nanomolar range were detected in the ligand binding assays. Nevertheless, the K_d value obtained for AVP was also equivalent to the range of K_d values obtained for binding assays of kidney membranes (Jard, 1983, supra; Jard, 1987, supra).

The K_H value for AII obtained here was equivalent, if not 10-fold lower, than the K_H values obtained in membrane binding assays done in the absence or presence of guanine nucleotides (Crane et al., 1982, supra; Rogers et al., supra 1986) and the K_H value obtained in the binding assay of intact cells using radiolabeled antagonist (Rogers et al., 1986, supra).

The level of expressed functional AII/AVP receptors in Cos A1/V9 cells was comparable to, if not better than, the levels of AII and/or AVP receptors (measured separately) in other cell lines or tissues. As deduced from our data, with 10^6 cells = 20 ug membrane protein (by actual measurements), the B_{max} for AVP_{V2} receptors in Cos A1/V9 cells was 300 fmol/mg membrane protein. This was comparable to MDCK cells where the B_{max} = 500; in LLC-PK1 cells where the B_{max} = 191 (Jans et al., *J. Biol. Chem.* 265:15379, 1990); and in somatic hybrid cells where the B_{max} = 21-47 (Jans et al., 1990, supra). Similarly, the B_{max} value for ^{125}I-AII binding in Cos A1/V9 cells of 354 fmol/mg membrane protein was at the median of the range of published B_{max} value for AII binding (i.e., 35 - 1300 fmol/mg membrane protein) obtained from rat tissues and primary cell lines (Gunther et al., *Circ. Res.* 47:278, 1980; Campanile et al., *J. Biol. Chem.* 257:4951, 1982; Rogers et al, 1986, supra; Douglas, *Am. J. Physiol.* 253:F1, 1987; Bouscarel et al., *J. Biol. Chem.* 263:14913, 1988; Grove and Speth,

Endocrinology 125:223, 1989), and from neuroblastoma-glioma hybrid cells (Carrithers et al., *Biochem. Biophys. Res. Comm.* 165:196, 1990).

Furthermore, the number of A1/V9 cDNA encoded AII/AVP_{V2} receptors expressed in Cos A1/V9 cells measured by either AII or AVP binding ranged from 3.5 to 4.2 x 10^3 receptors per cell. This, again, was comparable to the number of expressed cDNA-encoded serotonin 1c receptor in mouse fibroblast 3T3 cells (Julius et al., *Science* 241:558, 1988). In addition, the levels of AII- and AVP-induced cAMP accumulation in Cos A1/V9 cells (i.e., 125 and 275 pmol/mg membrane protein/ minute respectively) were within the range obtained in the analysis of $AVP_{V2-type}$ receptors in MDCK cells, (i.e., 46 pmol/mg membrane protein/min; (Friedlander and Amiel, *Biochem. Biophys. Acta* 929:311, 1987); in somatic cell hybrid cells (i.e., 0.89 to 8.34 pmol/mg membrane protein/min Jans et al., 1990, supra); and in LLC/PK1 cells, (i.e., 653 pmol/mg membrane protein/ min (Jans et al., 1990, supra).

Because of the novelty of the dual peptide ligand/single receptor system, competition by AII and AVP for the other's specific binding was investigated as follows.

Competition curves of ^{3}H-AVP binding using intact Cos A1/V9 cells were performed as described above with the following specifications: confluent cell cultures (in P-35 dishes) were incubated for 20 minutes at 37°C with 5nM ^{3}H-AVP (i.e., at K_d =5nM; Fig. 6) in the presence of increasing concentrations of competitor. Competition for ^{3}H-AVP specific binding to intact Cos A1/V9 cells by various AVP analogs and AII (♦) is presented in Fig. 9; competition by unlabeled AVP (■) is presented for comparison. The AVP analogs included the V2 agonist, DVDAVP (□), (Manning et al., *J. Med. Chem.* 16:975, 1973); the VI antagonist, [d(CH2)$_5$, Tyr(Me)]-AVP (△) (Kruszynski

et al., *J. Med. Chem.* 23:364, 1980); and the V1/V2 antagonist, [d(CH_2)$_5$, D-Ile^2, Ile^4]-AVP (▲) (Manning et al., *J. Med. Chem.* 27:423, 1984). Values for respective affinities (K_d and K_i) are presented in Fig. 6. Results in Figs. 9 and 6 represent three separate experiments with each point done in duplicate. The percent variation was 0.7-14%; mean percent variation was 6.5%.

As seen in Fig. 9, 10 μM AII did not displace AVP binding. Conversely, 10 μM AVP did not displace ^{125}I-AII binding (not shown). This was again consistent with the hypothesis of two discrete and independent binding sites for AII and AVP. The effective displacement of 5 nM ^{3}H-AVP, by [1-deamino, Val^4, D-Arg]-vasopressin (DVDAVP), a highly potent and specific antidiuretic AVP analog (Figs. 9 and 6) supported the V2-type characteristic of this kidney-derived AVP receptor as one functionally coupled to the adenylate cyclase system. Consistently, the specific V1-type receptor antagonist, [β-mercaptol β,β cyclopenta-methylenepropionyl^1, -O-Me-Tyr^2, Arg^8]-AVP, abbreviated [d(CH2)$_5$, Tyr(Me)]-AVP, exhibited markedly less displacement, (Figs. 9 and 6). The displacement of 5 nM ^{3}H-AVP by the V1/V2 antagonist, [d(CH_2)$_5$, D-Ile^2, Ile^4]-AVP was less effective than that exhibited by DVDAVP and slightly more effective than that by the V1-specific antagonist (Fig. 9). 100 nM of this V1/V2 antagonist completely displaced binding of 1nM ^{3}H-AVP, consistent with the amount used to block AVP-induced cAMP accumulation (Fig. 4A).

The A1/V9 cDNA was sequenced as follows. Single strand M13 templates of overlapping restriction digest fragments (in both orientations) were sequenced using the dideoxy chain termination method of Sanger et al. (*Proc. Natl. Acad. Sci. USA* 74: 5463, 1977) and Messing et al. (*Nucl. Acids. Res.* 9:309, 1981).

Nucleotide sequence analysis of the A1/V9 cDNA revealed a single long open reading frame encoding a protein of 481 amino acids, with a predicted molecular weight of 53,350 Kd. The nucleic acid sequence and deduced amino acid sequence are shown in Fig. 1 (SEQ ID NO:1). The predicted molecular weight approximated the apparent molecular weight from photoaffinity labeling, chemical crosslinking, and ligand affinity blotting studies (Fahrenholz et al., *Eur. J. Biochem.* 182:589, 1985; Fahrenholz et al., *J. Recep. Res.* 8:283, 1988; Marie and Roy, *Mol. Pharmacol.* 33:432, 1988). The sequence possessed a single region of high homology for each probe sequence: 4/8 amino acids (amino acids $_{392-399}$) for AII, and 4/9 amino acids (amino acids $_{342-350}$) for AVP. Likewise, at the nucleotide level the regions of highest homology (58%) to the AII and (78%) to the AVP oligonucleotide probes occurred only once and corresponded to the amino acid regions homologous to the AII and AVP probes. Regions posessing homology with the AVP cRNA oligonucleotide probe (1) and homology with the AII cRNA oligonucleotide probe (2) are marked by brackets in Fig. 1 (SEQ ID NO: 1); identical nucleotides are dotted.

Hydropathy analysis of the AII/AVP$_{V2}$ receptor by the method of Kyte and Doolittle (Kyte and Doolittle, *J. Mol. Biol.* 157:105, 1982), with a window of 20 (Engleman et al., *Ann. Rev. Biophys. Chem.* 15:321, 1986), predicted 7 putative transmembrane domains as delineated by 7 hydrophobic regions (marked H1-H7 in Fig. 1; SEQ ID NO: 1). Fig. 10 depicts the hydropathy profile of the AII/AVP$_{V2}$ receptor polypeptide. The hydropathy index is noted on the y-axis and the number of the central amino acid in the 20-amino acid window is noted on the x-axis. The first putative membrane spanning region may be longer than the predicted 17 aa-long H_1, however, the length of

this α-helix was sufficient to span the plasma membrane (Adams and Rose, *Cell* 41:1007, 1985). H_2 - H_7 were also of sufficient length to span the plasma membrane (Adams and Rose, 1985, supra).

Comparison of the AII/AVP_{V2} receptor sequence with known G protein-coupled non-peptide cationic ligand receptor sequences showed no significant homology. These results suggested that the AII/AVP_{V2} receptor most likely belonged to a new subclass of the superfamily of G protein coupled receptors, as expected considering the distinction that the AII/AVP_{V2} receptor is a small peptide ligand receptor and not a cationic agonist receptor nor a heterodimer glycoprotein hormone receptor.

A stretch of 12 consecutive negatively-charged amino acids was found to be located between H3 and H4 (i.e., amino acids 202-213) and a stretch of eight consecutive charged amino acids was found to be located between H4 and H5 (i.e., amino acids 282-289). In addition, the AII/AVP_{V2} receptor possessed charged amino acids in all seven predicted hydrophobic regions. Helical wheel analysis of H1-H7 revealed amphipathic putative transmembrane domains consistent with a channel-like or transporter-like structure (Krupinski et al., *Science* 244:1558, 1990). This was consistent with the putative involvement of this kidney AII/AVP_{V2} receptor in an AVP-sensitive water channel in kidney epithelial cells.

Finally, serine (i.e., S) residues within protein kinase C phosphorylation consensus sequences (Blackshear et al., *FASEB J.* 2:2957, 1988) were found to be located in the cytoplasmic loop between H4 and H5 and are circled in Fig. 1 (SEQ ID NO: 1).

Fig. 11 depicts the putative structure of the AII/AVP_{V2} receptor. Based on the localization of the 7 hydrophobic regions (H1-7) as putative transmembran

domains (depicted as barrels through the stippled plasma membrane), the putative AVP binding site [with identical amino acids (•) to the antipeptide probe sequence and conservative amino acid substitutions (o) indicated] in the loop between H5 and H6, and the putative AII binding site within the N-terminus of H6, a putative structure was determined with the N-terminus intracellularly and the C-terminus extracellularly. Potential phosphorylation sites are marked (*). The charged amino acids in the transmembrane domains and the stretch of 12 consecutive negatively charged amino acids in the loop between H4 and H5 are indicated (-) and (+), respectively. Basic amino acids flanking serine residues (S) and comprising the putative phosphorylation sites (Blackshear et al., 1988, supra) are marked (+).

The AII and AVP peptide binding sites were found in proximity to each other in the region between H5 and H6 (Fig. 1; SEQ ID NO:1).

Cloning of the Human AII/AVP_{V2} Receptor Gene

Isolation of the rat AII/AVP_{V2} receptor gene facilitates the isolation of the human AII/AVP_{V2} gene. A probe is designed based on the rat AII/AVP_{V2} gene sequence (Fig. 1; SEQ ID NO: 1) and used to probe a human kidney λgt11 cDNA library (e.g., obtained from Clontech, Palo Alto, CA); such a probe preferably includes the entire 2.25 kb AII/AVP_{V2} receptor-encoding fragment. Hybridization is carried out under low stringency conditions, specifically, using a hybridization buffer containing 5X SSPE, 0.1% SDS, 0.2 mg/ml calf thymus DNA, 1% bovine serum albumin, 1% polyvinyl pyrrolidine (PVP), 1% Ficoll, and 10% formamide, at 37°C for 24 hours. Hybridization is followed by three washes in 2X SSPE and 0.1% SDS at 45°C for 15 minutes each. Hybridizing plaques are preferably purified 4 times. Probe preparation, hybridization, and plaque purification are

carri d out as described in Ausub l et al. (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1989). A putative human AII/AVP_{V2} receptor-encoding cDNA is verified by DNA sequencing (and comparison with the rat homologue described herein) and by expression in mammalian cells followed by receptor binding and functional assays as described herein.

Identification and Isolation of an AII/AVP_{V2} Mutant in Hypertensive Rats

A cDNA library was prepared from Dahl-salt sensitive hypertensive rats (DS rats) as described in Herrera and Ruiz-Opazo (*Science* 249:1023, 1990). This library was screened, using as a hybridization probe, a 1.3 kb PstI/BglII fragment of the A1/V9 cDNA (i.e., coding for amino acids 30 through 466 of Fig. 1; SEQ ID NO. 1). Hybridization was carried out under low stringency, specifically, in a hybridization buffer containing: 5X SSPE, 0.1% SDS, 0.2 mg/ml calf thymus DNA, 1% BSA, 1% PVP, 50% formamide, at 37°C for 24 hours. Hybridization was followed by three washes in 2X SSPE and 0.1% SDS at 45° for 15 minutes each. A cDNA encoding a full length AII/AVP_{V2} receptor was isolated from the DS rat library, and characterized by nucleic acid sequencing (as described above). This clone, termed C/R_{163}, possessed a nucleic acid substitution (i.e., a T for a C) at nucleotide 487 resulting in an amino acid substitution (i.e., an arginine for a cysteine) at amino acid 163.

An 875 bp KpnI/BstXI fragment including the C/R_{163} cDNA was then excised from recombinant plasmid pSP73-C/R_{163} and inserted into the backbone of a KpnI/BstXI-digested pMAMneo-A1/V9 (wild-type) vector (termed pMAM-DR-AII/AVP_{V2}) to produce plasmid pMAM-DS-AII/AVP_{V2}.

Both the wild-type "DR" expression plasmid (i.e., pMAM-DR-AII/AVP_{V2}) and the mutant "DS" expression plasmid (i.e., pMAM-DS-AII/AVP_{V2}) were transfected into C-127

cells (i.e., 60 μg plasmid DNA/10^7 cells) and, five days post-transfection, crude membranes were prepared from the cells by the method of Takuwa et al. (*J. Clin. Invest.* 85:653, 1990), and 10μg of DS and DR AII/AVP_{V2} receptor-expressing membranes were independently exposed to either 0.1 μM AVP or 0.1 μM AII at 25°C for 20 minutes (as described above) in the presence of 0, 50, or 150mM NaCl. Results were compared to a basal control (i.e., with no hormone added), and each point was performed in duplicate. The mean percent variation was 3.5% for the AVP induction experiments and 2.5% for the AII induction experiments.

As shown in Fig. 12 and Fig. 13, at 50mM NaCl, the response to AVP and AII differs significantly between the wild type DR-AII/AVP_{V2} receptor (i.e., pMRA1V9 in Fig. 12 and DR in Fig. 13) and the mutant DS-AII/AVP_{V2} receptor (i.e., pMSA1V9 in Fig. 12 and DS in Fig. 13); the DS-AII (AVP_{V2} receptor exhibited a 2-fold and 3-fold greater response to AVP and AII, respectively. These differences in cAMP accumulation were not due to differences in receptor number since at 0 mM NaCl, similar AII and AVP activities were observed (Fig. 12). In addition, G-protein abnormality was ruled out by use of the C127 transient cell expression system. These results suggest that the C/R_{163} mutation in the DS-AII/AVP_{V2} receptor results in an altered coupling to adenylate cyclase.

Screening for Humans with Susceptibility to Hypertension

Isolation of a mutation in the rat AII/AVP_{V2} receptor which correlates with hypertension facilitates a screen which is used to identify human patients who are afflicted with hypertension or who are likely to develop hypertension in the future. The screen is carried out as follows.

DNA from a human patient is isolated from blood cells as described in Innis et al. (*PCR Protocols: A*

Guide to Methods and Applications, Academic Press). Polymerase chain reaction (PCR) primers are obtained commercially or synthesized using a Dupont (Willmington, DE) or Applied Biosystems (Foster City, CA) oligonucleotide synthesizer and the instructions of the supplier. The sequence of the oligonucleotide primers correspond to sequences flanking the Cys_{163}-containing exon of the human receptor gene; this particular exon is identified by sequence homology with the rat sequence (above). The primers are annealed to the isolated human DNA and PCR carried out by the techniques of Innis et al. (above). The PCR-amplified DNA is then sequenced (as described above), and sequences examined for those exhibiting a mutation at the human amino acid corresponding to rat AII/AVP_{V2} receptor amino acid 163. A patient whose AII/AVP_{V2} receptor DNA possesses such a mutation is diagnosed either as being hypertensive or as having a propensity toward developing hypertension.

Alternatively, allele-specific amplification (as described in Innis et al., above) of a DNA sequence containing a human "Cys_{163}" mutation is used to identify individuals who are, or who are likely to become, hypertensive.

Polypeptide Expression

Polypeptides according to the invention may be produced by transformation of a suitable host cell with all or part of an AII/AVP_{V2} receptor-encoding cDNA fragment (e.g., the cDNAs described above) in a suitable expression vehicle.

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used to provide the recombinant receptor prot in. The precise host cell used is not critical to the invention, however the following host cells are preferred: COS 1 and C127 cells. Such cells ar

available from a wide range of sources (e.g., the American Type Culture Collection, Rockville, MD; ATCC Accession Nos. CRL 1650 and CRL 1616, respectively). The method of transfection and the choice of expression vehicle will depend on the host system selected. Mammalian cell transfection methods are described, e.g., in Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1989); expression vehicles may be chosen from those provided, e.g., in *Cloning Vectors: A Laboratory Manual* (P.H. Pouwels et al., 1985, Supp. 1987).

One preferred expression system is the mouse 3T3 fibroblast host cell transfected with a pMAMneo expression vector (Clontech, Palo Alto, CA). pMAMneo provides: an RSV-LTR enhancer linked to a dexamethasone-inducible MMTV-LTR promotor, an SV40 origin of replication which allows replication in mammalian systems, a selectable neomycin gene, and SV40 splicing and polyadenylation sites. DNA encoding the human or rat AII/AVP_{V2} receptor or an appropriate receptor fragment or analog (as described above) is inserted into the pMAMneo vector in an orientation designed to allow expression. The recombinant receptor protein is isolated as described below. Other preferable host cells which may be used in conjunction with the pMAMneo expression vehicle include COS cells, CHO cells, and C127 cells (ATCC Accession Nos. CRL 1650, CCL 61, and CRL1616, respectively).

Alternatively, the human or rat AII/AVP_{V2} receptor (or receptor fragment or analog) is produced by a stably-transfected mammalian cell line.

A number of vectors suitable for stable transfection of mammalian cells are available to the public, e.g., see Pouwels et al. (supra); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (supra). In one example, cDNA

encoding the receptor (or receptor fragment or analog) is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the AII/AVP_{V2} receptor encoding gene into the host cell chromosome is selected for by inclusion of 0.01-300 μM methotrexate in the cell culture medium (as described in Ausubel et al., supra). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHRF and pAdD26SV(A) (described in Ausubel et al., supra). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO $DHFR^-$ cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

Once the recombinant AII/AVP_{V2} receptor protein (or fragment or analog, thereof) is expressed, it is isolated, e.g., using affinity chromatography. In one example, AII, AVP, or an anti-AII/AVP_{V2} receptor antibody (e.g., produced as described below) may be attached to a column and used to isolate intact receptor or receptor fragments or analogues. Lysis and fractionation of receptor-harboring cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., supra). Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography (see, e.g., Fisher,

Laboratory Techniques In Biochemistry And Molecular Biology, eds., Work and Burdon, Elsevier, 1980).

Receptors of the invention, particularly short receptor fragments, can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL).

Assays for AII/AVP_{V2} Receptor Binding and Function

Useful receptor fragments or analogues in the invention are those which interact with AII or AVP. Such an interaction may be detected by an in vitro binding assay (described herein). The receptor component may also be assayed functionally, i.e., for its ability to bind AII or AVP and mediate an increase in intracellular cAMP (described herein). These assays include, as components, AII or AVP and a recombinant AII/AVP_{V2} receptor (or a suitable fragment or analog) configured to permit detection of binding.

AII and AVP may be obtained from Sigma (St. Louis, MO).

Preferably, the AII/AVP_{V2} receptor component is produced by a cell that naturally presents substantially no receptor, e.g., by engineering such a cell to contain nucleic acid encoding the receptor component in an appropriate expression system. Suitable cells are, e.g., those discussed above with respect to the production of recombinant receptor, such as COS 1 cells or C127 cells.

The binding assay is preferably performed by isolating membranes from recombinant cells expressing the AII/AVP_{V2} receptor protein and detecting specific binding of a radiolabelled ligand as label in association with the membrane preparation.

The assay may also be performed by fixing the r combinant cell expressing the AII/AVP_{V2} receptor component to a solid substrate (e.g., a test tube, a

microtiter well, or a column) by means well known to those in the art (see, e.g., Ausubel et al., supra) and presenting labelled AII or AVP (e.g., ^{3}H-labelled AVP or ^{125}I-labelled AII) to the immobilized cells. Binding is assayed by the detection of label in association with the receptor component (and, therefore, in association with the solid substrate).

In this assay, the format may be any of a number of suitable formats for detecting specific binding, such as a radioimmunoassay format (see, e.g., Ausubel et al., supra). Preferably, cells transiently or stably transfected with an AII/AVP_{V2} receptor expression vector (see above) are immobilized on a solid substrate (e.g., the well of a microtiter plate) and reacted with AII or AVP which is detectably labelled, e.g., with a radiolabel or an enzyme which can be assayed, e.g., alkaline phosphatase or horseradish peroxidase.

Alternatively, binding may be detected using a related assay. AII or AVP may be adhered to a solid substrate (e.g., a microtiter plate using methods similar to those for adhering antigens for an ELISA assay; Ausubel et al., supra) and the ability of labelled AII/AVP_{V2} receptor-expressing cells (e.g., labelled with ^{3}H-thymidine; Ausubel et al., supra) can be used to detect specific receptor binding to the immobilized AII or AVP.

In one particular example, a vector expressing the AII/AVP_{V2} receptor (or receptor fragment or analog) is transfected into Cos 1 or C127 cells by the DEAE dextran-chloroquine method (Ausubel et al., supra). Expression of the receptor protein confers binding of detectably-labelled AII or AVP to the cells. Neither AII nor AVP binds significantly to untransfected host cells or cells bearing the parent vector alone; these cells ar used as a "control" against which the binding assays are

measured. 10 cm. tissue culture dishes are seeded with AII/AVP_{V2} receptor-expressing Cos 1 or C127 cells (approximately 750,000 cells, dish) 12-18h post-transfection. Forty-eight hours later, triplicate dishes are incubated with radiolabelled AII (0.1 mM) or AVP (5mM) (e.g., ^{125}I-AII or ^{3}H-AVP) and binding to the receptor-bearing cells is assayed (e.g., by harvesting the cells and assaying the amount of detectable label in association with the cells).

In all of the above assays, cells (or membranes of such cells) which specifically bind labelled AII or AVP are those which exhibit a level of binding (i.e., an amount of detectable label) which is greater than that of the control cells (or membranes).

A recombinant receptor may also be assayed functionally for its ability to mediate an AII or AVP and AII/AVP_{V2} receptor-dependent increase in intracellular cAMP. Cells, preferably Cos 1 cells transfected with an AII/AVP_{V2} receptor expression vector, are assayed for intracellular cAMP levels as described herein. A recombinant receptor which promotes an increased level of intracellular cAMP upon AII or AVP treatment (as measured herein) are receptors useful in the invention.

Screening For AII/AVP_{V2} Receptor Antagonists

As discussed above, one aspect of the invention features screening for compounds that antagonize the interaction between AII or AVP and the AII/AVP_{V2} receptor, thereby preventing or reducing the cascade of events that are mediated by that interaction. The elements of a screen to identify antagonists are AII or AVP and recombinant AII/AVP_{V2} receptor (or a suitable receptor fragment or analog, as outlined above) configured to permit detection of binding. AII and AVP are publically available from Sigma (see above). Full-length rat or human AII/AVP_{V2} receptor protein (or an AII- or AVP-

binding fragment or analog) may be produced as described herein.

Binding of AII or AVP to its receptor may be assayed by any of the methods described above.

Preferably, cells expressing recombinant AII/AVP_{V2} receptor (or a suitable AII/AVP_{V2} receptor fragment or analogue) are immobilized on a solid substrate (e.g., the well of a microtiter plate or a column) or membranes including recombinant protein are isolated and reacted with detectably-labelled AII or AVP (as described above). Binding is assayed by the detection label in association with the receptor component (and, therefore, in association with the solid substrate or membrane). Binding of labelled AII or AVP to receptor-bearing cells is used as a "control" against which antagonist assays are measured. The antagonist assays involve incubation of the AII/AVP_{V2} receptor-bearing cells with an appropriate amount of candidate antagonist. To this mix, an equivalent amount of labelled AII or AVP is added. An AII or AVP antagonist useful in the invention specifically interferes with labelled AII or AVP binding to the immobilized receptor-expressing cells.

An antagonist is then tested for its ability to interfere with AII/AVP_{V2} receptor function, i.e., to specifically interfere with labelled AII/AVP_{V2} receptor:ligand binding without resulting in the signal transduction normally mediated by the ligand. These properties of useful antagonists are tested using the functional assay described herein. Specifically, Cos 1 cells expressing the recombinant receptor are reacted with AII or AVP, and the intracellular cAMP levels are measured. This is considered to be a "control" level. Addition of potential antagonists along with, or just prior to addition of, AII or AVP allows for the screening and identification of authentic receptor antagonists.

Such an antagonist prevents the AII- or AVP-mediated increase in cAMP levels.

Appropriate candidate antagonists include AII/AVP_{V2} receptor fragments, particularly fragments containing an AII- or AVP-binding portion, e.g., amino acids 392-399 and amino acids 342-350 (described above); such fragments preferably include five or more amino acids. Other candidate antagonists include analogues of AII or AVP and other peptides as well as non-peptide compounds designed or derived from analysis of the receptor and anti-AII/AVP_{V2} receptor antibodies.

Anti-AII/AVP_{V2} Receptor Antibodies

Human or rat AII/AVP_{V2} receptor (or immunogenic receptor fragments or analogues) may be used to raise antibodies useful in the invention. As described above, receptor fragments preferred for the production of antibodies are those fragments deduced or shown experimentally to be extracellular.

Antibodies directed to AII/AVP_{V2} receptor peptides are produced as follows. Peptides corresponding to the AII- or AVP-binding portion (e.g., amino acids 392-399 and 342-350, respectively) or to all or part of a putative extracellular domain (i.e., amino acids 30 to 94, amino acids 151 to 251, amino acids 338 to 390, and amino acids 437 to 481, and preferably, amino acids 193-200 of Fig. 1; SEQ ID NO: 1) are produced using a peptide synthesizer, by standard techniques (see, e.g., *Solid Phase Peptide Synthesis*, supra; Ausubel et al., supra) or by recombinant means (Ausubel et al., supra). The peptides may be coupled to a carrier protein, such as KLH as described in Ausubel et al, supra. The KLH-peptide is mixed with Freund's adjuvant and injected into guinea pigs, rats, or preferably rabbits. Antibodies are purified by peptide antigen affinity chromatography.

Once produced, antibodies are tested for specific AII/AVP_{V2} receptor recognition by Western blot or immunoprecipitation analysis (by the methods described in Ausubel et al., supra). Antibodies which specifically recognize the AII/AVP_{V2} receptor are considered to be candidates for useful antagonists; such candidates are further tested for their ability to specifically interfere with the interaction between AII or AVP and the AII/AVP_{V2} receptor (as described above) or AII/AVP_{V2} receptor function (as described above). Antibodies which antagonize AII:AII/AVP_{V2} receptor binding or AVP:AII/AVP_{V2} receptor binding or AII/AVP_{V2} receptor function are considered to be useful as antagonists in the invention.

Therapy

Therapeutics for the treatment of hypertension are the soluble antagonist receptor fragments described above formulated in an appropriate buffer such as physiological saline. Where it is particularly desirable to mimic the receptor conformation at the membrane interface, the fragment may include a sufficient number of adjacent transmembrane residues. In this case, the fragment may be associated with an appropriate lipid fraction (e.g., in lipid vesicles or attached to fragments obtained by disrupting a cell membrane). Alternatively, anti-AII/AVP_{V2} receptor antibodies produced as described above may be used as a therapeutic. Again, the antibodies would be administered in a pharmaceutically-acceptable buffer (e.g., physiological saline). If appropriate, the antibody preparation may be combined with a suitable adjuvant.

The therapeutic preparation is administered in accordance with the condition to be treated. Ordinarily, it will be administered intravenously, at a dosage that provides suitable competition for AII or AVP binding. Alternatively, it may be convenient to administer the

therapeutic orally, nasally, or topically, e.g., as a liquid or a spray. Again, the dosages are as described above. Treatment may be repeated as necessary for alleviation of disease symptoms. Antagonists may also be administered to prevent (as well as treat) hypertension; the antagonist is administered as described above.

Because both AII and AVP binding to the AII/AVP_{V2} trigger receptor function (as indicated by increased intracellular cAMP concentrations), it may be preferable to administer an antagonist which interferes with binding of both AII and AVP or, alternatively, to administer a combination of antagonists, including one which interferes with AII binding and one which interferes with AVP binding. Such antagonists or combinations of antagonists are tested for efficacy using the assays described herein and are administered as described above.

Because the AII/AVP_{V2} receptor is likely involved in AII- and AVP-mediated control of blood vessel contraction, AII/AVP_{V2} receptor antagonists can be used to treat or prevent disorders such as hypertension and related illness (e.g., stroke triggered by hypertension).

The methods of the invention may be used to reduce the disorders described herein in any mammal, for example, humans, domestic pets, or livestock. Where a non-human mammal is treated, the AII/AVP_{V2} receptor or receptor fragment or analog or the antibody employed is preferably specific for that species.

Other embodiments are within the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: the Trustees of Boston University

(ii) TITLE OF INVENTION: ANGIOTENSIN II_{cAMP}/ $VASOPRESSIN_{V2}$ RECEPTORS AND RELATED MOLECULES AND METHODS

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Fish & Richardson
(B) STREET: 225 Franklin Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: U.S.A.
(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM: IBM P.C. DOS (Version 3.30)
(D) SOFTWARE: WordPerfect (Version 5.0)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 07/758,921

(B) FILING DATE: September 11, 1991

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Clark, Paul T.

(B) REGISTRATION NUMBER: 30,162

(C) REFERENCE/DOCKET NUMBER: 04766/002WO1

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 542-5070

(B) TELEFAX: (617) 542-8906

(C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2296

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGCACTTACC GCTTCGTGAA AGAGAATGAG ACGCTGTACG CACTGTGCTT TGTGCCGTTT 60

GTGTGCTGGA TCGTGTGCAC CGTGCTGCTG CAGCAA 96

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ATG GAG CTG GGC CGG GAT CTG TCT CGT ACC TCC AAG ACC ACT ACA TCT          144
Met Glu Leu Gly Arg Asp Leu Ser Arg Thr Ser Lys Thr Thr Thr Ser
                5                  10                  15

GTG TAC CTG CTC TTC ATC ACC AGC ATG CTG AAG TCT GCA GGT ACC AAT          192
Val Tyr Leu Leu Phe Ile Thr Ser Met Leu Lys Ser Ala Gly Thr Asn
            20                  25                  30

GGA CCC CGG GTT CAG GGA GAG CTG CGA ATG CTG TGC CGC CTG GCC CGG          240
Gly Pro Arg Val Gln Gly Glu Leu Arg Met Leu Cys Arg Leu Ala Arg
        35                  40                  45

GAG GGC ATC CTG AAG CAT CAA GCA CAG TTC TCA GAA AAG GAC CTG GAG          288
Glu Gly Ile Leu Lys His Gln Ala Gln Phe Ser Glu Lys Asp Leu Glu
    50                  55                  60

AGA TTG AAG CTT CAG GGT TCC CAA GTT CAG ACA ATG TTT CTC AGC AAG          336
Arg Leu Lys Leu Gln Gly Ser Gln Val Gln Thr Met Phe Leu Ser Lys
65                  70                  75                  80

AAG GAG CTG CCA GGA GTG CTA GAA ACT GTG GTC ACC TAC CAG TTC ATT          384
Lys Glu Leu Pro Gly Val Leu Glu Thr Val Val Thr Tyr Gln Phe Ile
                85                  90                  95

GAC CAG AGC TTC CAG GAG TTC TTG GCT GCA TTG TCA TAC CTA CTA GAC          432
Asp Gln Ser Phe Gln Glu Phe Leu Ala Ala Leu Ser Tyr Leu Leu Asp
            100                 105                 110
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GCT GAG GGA GCC CCA GGG AAC TCC GCA GGA AGT GTG CAG ATG CTC CTG          480
Ala Glu Gly Ala Pro Gly Asn Ser Ala Gly Ser Val Gln Met Leu Leu
        115                 120                 125

AAC TCT GAC GCG GGG CTG CGT GGT CAT CTG GCA CTC ACC ACT ...  TTG          528
Asn Ser Asp Ala Gly Leu Arg Gly His Leu Ala Leu Thr Thr Arg Phe
    130                 135                 140

CTC TTT GGA CTG CTA AGT ACA GAG AGG ATT CGT GAC ATT GGA AAC CAT          576
Leu Phe Gly Leu Leu Ser Thr Glu Arg Ile Arg Asp Ile Gly Asn His
145                 150                 155                 160

TTT GGC TGT GTG GTG CCA GGG CGT GTG AAA CAG GAC ACC TTG CGG TGG          624
Phe Gly Cys Val Val Pro Gly Arg Val Lys Gln Asp Thr Leu Arg Trp
                165                 170                 175

GTA CAA GGA CAA AGC CAA CCC AAG GTG GCG ACA GTA GGA GCA GAA AAG          672
Val Gln Gly Gln Ser Gln Pro Lys Val Ala Thr Val Gly Ala Glu Lys
            180                 185                 190

AAG GAT GAG CTG AAG GAC GAG GAA GCA GAG GAG GAG GAG GAG GAG GAA          720
Lys Asp Glu Leu Lys Asp Glu Glu Ala Glu Glu Glu Glu Glu Glu Glu
        195                 200                 205

GAA GAG GAG GAG GAA CTC AAC TTT GGA CTG GAG CTG TTG TAC TGC CTG          768
Glu Glu Glu Glu Glu Leu Asn Phe Gly Leu Glu Leu Leu Tyr Cys Leu
    210                 215                 220

TAT GAG ACA CAA GAG GAT GAT TTT GTT CGC CAG GCT CTC AGC AGC CTT          816
Tyr Glu Thr Gln Glu Asp Asp Phe Val Arg Gln Ala Leu Ser Ser Leu
225                 230                 235                 240

CCA GAG ATG GTA CTG GAG CGA GTT AGG CTG ACC CGC ATG GAC CTT GAG          864
Pro Glu Met Val Leu Glu Arg Val Arg Leu Thr Arg Met Asp Leu Glu
                245                 250                 255

GTT CTG AGC TAC TGC GTG CAG TGC TGC CCG GAC GGC CAG GCT CTG AGA          912
Val Leu Ser Tyr Cys Val Gln Cys Cys Pro Asp Gly Gln Ala Leu Arg
            260                 265                 270
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CTG GTG AGC TGT GGA CTG GTG GCG GCA AAG GAG AAG AAG AAG AAG AAG          960
Leu Val Ser Cys Gly Leu Val Ala Ala Lys Glu Lys Lys Lys Lys Lys
        275                 280                 285

AAG AGC TTC ATG AAC CGG CTG AAG GGT TCT CAA AGC ACC GGG AAA CAA         1008
Lys Ser Phe Met Asn Arg Leu Lys Gly Ser Gln Ser Thr Gly Lys Gln
    290                 295                 300

CCC CCA GCC TCC TTG CTG CGT CCA CTC TGT GAG GCA ATG ATT ACC CAG         1056
Pro Pro Ala Ser Leu Leu Arg Pro Leu Cys Glu Ala Met Ile Thr Gln
305                 310                 315                 320

CAA TGT GGT CTG AGT ATT CTG ACC TTG TCA CAC TGC AAA CTC CCT GAT         1104
Gln Cys Gly Leu Ser Ile Leu Thr Leu Ser His Cys Lys Leu Pro Asp
                325                 330                 335

GCA GTT TGT CGA GAC CTT TCT GAG GCT CTG AAG GTA GCT CCT TCC CTA         1152
Ala Val Cys Arg Asp Leu Ser Glu Ala Leu Lys Val Ala Pro Ser Leu
            340                 345                 350

AGG GAG CTG GGC CTC CTC CAG AAC CGA CTC ACT GAG GCA GGG CTG CGT         1200
Arg Glu Leu Gly Leu Leu Gln Asn Arg Leu Thr Glu Ala Gly Leu Arg
        355                 360                 365

TTA CTA AGC CAA GGC CTG GCT TGG CCC AAA TGC AAG GTG CAG ACA CTC         1248
Leu Leu Ser Gln Gly Leu Ala Trp Pro Lys Cys Lys Val Gln Thr Leu
    370                 375                 380

AGG ATA CAG ATG CCT GGG CTC CAA GAG GTG ATC CAC TAC CTG GTC ATT         1296
Arg Ile Gln Met Pro Gly Leu Gln Glu Val Ile His Tyr Leu Val Ile
385                 390                 395                 400

GTG CTC CAG CAG AGC CCA GTC CTA ACC ACT CTG GAC CTC AGT GGC TGT         1344
Val Leu Gln Gln Ser Pro Val Leu Thr Thr Leu Asp Leu Ser Gly Cys
                405                 410                 415

CAG CTG CCT GGG ACT GTG GTG GAA CCT CTG TGT TCA GCC CTG AAG CAC         1392
Gln Leu Pro Gly Thr Val Val Glu Pro Leu Cys Ser Ala Leu Lys His
            420                 425                 430
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CCT AAA TGT GGC CTA AAG ACC CTC AGT CTG ACT TCT GTG GAG CTG ACT          1440
Pro Lys Cys Gly Leu Lys Thr Leu Ser Leu Thr Ser Val Glu Leu Thr
        435                 440                 445
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GAG AAT CCA CTG AGA GAG CTT CAA GCT GTG AAG ACA TTA AAG CCA GAT          1488
Glu Asn Pro Leu Arg Glu Leu Gln Ala Val Lys Thr Leu Lys Pro Asp
    450                 455                 460

CTG GCC ATC ATA CAT TCA AAA TTG GGC ACA CAT CCT CAG CCT CTG AAG          1536
Leu Ala Ile Ile His Ser Lys Leu Gly Thr His Pro Gln Pro Leu Lys
465                 470                 475                 480

GGA                                                                      1584
Gly

TGAAGCAGTG TCTTCTGAGC CCCGGAGTCC AGAACAGGAT ATTGCTGAAG TCAACGTTCT        1644

TCTGAATATG TCTCCCCATT CAAAAGCAAG TAGATGCCAG CTTCTCCCTC CAGGCAAGTC        1704

TTTCAAGCCA AGAAGCCACA GAAGGGCAAG CAAAAGACCC AGGTAGATAT AGGCACTTAA        1764

GAGCCCCTGG ATATAGACCT GGCACATGTC CTGCCCCGGA CACTCGGAGG TTAGTCTTCC        1824

CTCAGCCCCA TAACCGCCAA TACCTCCCTT TCTGGGCCCA CCAATCTGTC CCTTGAAGAT        1884

AATCCCAACA GTAACAGAAG TATTTGTTGT GGTTTGCCCT GGAGTTATCT GTATTTTGAT        1944

GCTACTTCCA CTGCCCCAAG GACGGCTGCC TAGTCTGTAC TCAGGACTCG GGTGATTTCA        2004

CCAGAACCTT CTCCCTATTT AATTTGTAAA ATACCAATGA GGGCAGGTAC AAGATAGAAG        2064

GAGGCCTGTC ATTGGATGAG AAGAAAGGAT GGGTGGGAGA AACGTTTGAA GGAAGAGGAG        2124

GAGACTGGAC TGGAAAGGAG AAAGAGACAG GAGGGACAGA GAAAGTAGCC ATGGCGGGAC        2184

AATGTGGAAG CTGATGTTAA GATTCCAATA AGATCCCACG CTGTACCTTT ATAGGTTGTT        2244

ATGAATGTTC TTAAGGGATG GATGTGTATG GGGCTTTGTA TGTTTAGGTG GG                2296
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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AAAGGGGTGG ATGTATACGC GGTC 24

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TTCTCTTGGG CAGTTCTGGA AGTAGCA 27

Claims

1. Recombinant angiotensin II_{cAMP}/vasopressin$_{V2}$ (AII/AVP$_{V2}$) receptor polypeptide.

2. The polypeptide of claim 1, comprising an amino acid sequence substantially identical to the amino acid sequence shown in Fig. 1 (SEQ ID NO: 1).

3. A substantially pure polypeptide which is a fragment or analog of an AII/AVP$_{V2}$ receptor comprising a domain capable of binding angiotensin II (AII) or arginine-vasopressin (AVP).

4. The polypeptide of claim 1 or 3, wherein said receptor is derived from a mammal.

5. The polypeptide of claim 4, wherein said mammal is a human.

6. The polypeptide on claim 4, wherein said mammal is a rat.

7. A polypeptide comprising an AII-binding portion of an AII/AVP$_{V2}$ receptor.

8. The polypeptide of claim 7, comprising amino acids 392-399 of Fig. 1 (SEQ ID NO: 1).

9. A polypeptide comprising an AVP-binding portion of an AII/AVP$_{V2}$ receptor.

10. The polypeptide of claim 9, comprising amino acids 342-350 of Fig. 1 (SEQ ID NO: 1).

11. A polypeptide comprising an extracellular domain of an AII/AVP_{V2} receptor or an immunogenic analog thereof.

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12. The polypeptide of claim 11, comprising amino acids 30-94, amino acids 151-251, amino acids 338-390, or amino acids 437-481 of Fig. 1 (SEQ ID NO: 1), or an immunogenic analog thereof.

13. The polypeptide of claim 12, comprising amino acids 193-200 of Fig. 1 (SEQ ID NO: 1).

14. The polypeptide of claims 7, 9 or 11, further characterized in that said polypeptide is a recombinant polypeptide.

15. Purified DNA which encodes a polypeptide of claims 1, 3, 7, or 9.

16. The purified DNA of claim 15, wherein said DNA is cDNA.

17. The purified DNA of claim 15, wherein said DNA encodes a rat AII/AVP_{V2} receptor.

18. The purified DNA of claim 15, wherein said DNA encodes a human AII/AVP_{V2} receptor.

19. The purified DNA of claim 17, wherein said DNA is included in the plasmid pSVL-A1/V9.

20. The purified DNA of claim 17, wherein said DNA is included in the plasmid pMAM-DR-AII/AVP_{V2}.

21. A vector comprising the purified DNA of claim 15, said vector being capable of directing expression of the protein encoded by said DNA in a vector-containing cell.

22. A cell which contains the purified DNA of claim 15.

23. The cell of claim 22, said cell being a eukaryotic cell.

24. The cell of claim 23, said cell being a mammalian cell.

25. The cell of claim 24, said cell being a COS 1 cell or a C127 cell.

26. A method of producing a recombinant AII/AVP_{V2} receptor polypeptide or a fragment or analog thereof comprising,

providing a cell transformed with DNA encoding an AII/AVP_{V2} receptor or a fragment or analog thereof positioned for expression in said cell;

culturing said transformed cell under conditions for expressing said DNA; and

isolating said recombinant AII/AVP_{V2} receptor polypeptide.

27. A purified antibody which binds preferentially to a polypeptide of claims 1, 3, 7, 9, or 11.

28. The antibody of claim 27, wherein said antibody neutralizes <u>in vivo</u> a polypeptide of claims 1, 3, 7, or 9.

29. A method of testing a candidate compound for the ability to inhibit binding of AII to an AII/AVP_{V2} receptor, said method comprising:

a) contacting said candidate compound with a recombinant AII/AVP_{V2} receptor polypeptide of claim 1 or claim 3 and with AII;

b) measuring binding of said AII to said receptor polypeptide; and

c) identifying an antagonistic compound as one which decreases said binding.

30. A method of testing a candidate compound for the ability to inhibit binding of AVP to an AII/AVP_{V2} receptor, said method comprising:

a) contacting said candidate compound with a recombinant AII/AVP_{V2} receptor polypeptide of claim 1 or claim 3 and with AVP;

b) measuring binding of said AVP to said receptor polypeptide; and

c) identifying an antagonistic compound as one which decreases said binding.

31. The method of claim 29 or 30, wherein said receptor polypeptide is expressed on the surface of a recombinant cell.

32. The method of claim 29 or 30, wherein said candidate antagonist compound is further characterized as being capable of inhibiting the AII- or AVP-mediated increase in the intracellular cAMP concentration of a cell bearing said recombinant receptor on its surface.

33. A therapeutic composition comprising as an active ingredient a polypeptide according to claims 1, 3, 7, r 9, said active ingredient being formulat d in a physiologically-acceptable carrier.

34. The therapeutic composition of claim 33, wherein said polypeptide is anchored within the membrane of a cell.

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35. A therapeutic composition comprising as an active ingredient an antibody which neutralizes in vivo an AII/AVP_{V2} receptor, said active ingredient being formulated in a physiologically-acceptable carrier.

36. A method for identifying DNA associated with hypertension, said method comprising determining the sequence of the AII/AVP_{V2} receptor gene in said DNA, the presence of a mutation homologous to the rat C/R_{163} mutation being indicative of hypertension or a propensity toward hypertension.

37. The method of claim 36, wherein said DNA is amplified by polymerase chain reaction.

38. The method of claim 37, wherein said DNA amplification is allele specific.

39. A method for identifying an AII/AVP_{V2} receptor gene associated with hypertension, said method comprising expressing said gene in cultured cells, and determining the extent of AII-induced or AVP-induced cAMP accumulation in the presence of NaCl, an increased cAMP accumulation relative to wild-type being indicative of an association with hypertension.

(SEQ ID NO: 1)

FIGURE 2

AVP-induced cAMP accumulation in membranes prepared from RNA-microinjected Xenopus laevis oocytes.

RNA microinjected	experimental condition	adenylate cyclase activity (pmols/20 min/mg protein)
(-) A1/V9	AVP	26 ± 0.2
	NaF	75 ± 1.2
	control	25 ± 0.1
(+) A1/V9	AVP	56 ± 0.7
	NaF	83 ± 0.2
	control	28 ± 0.2

FIGURE 3

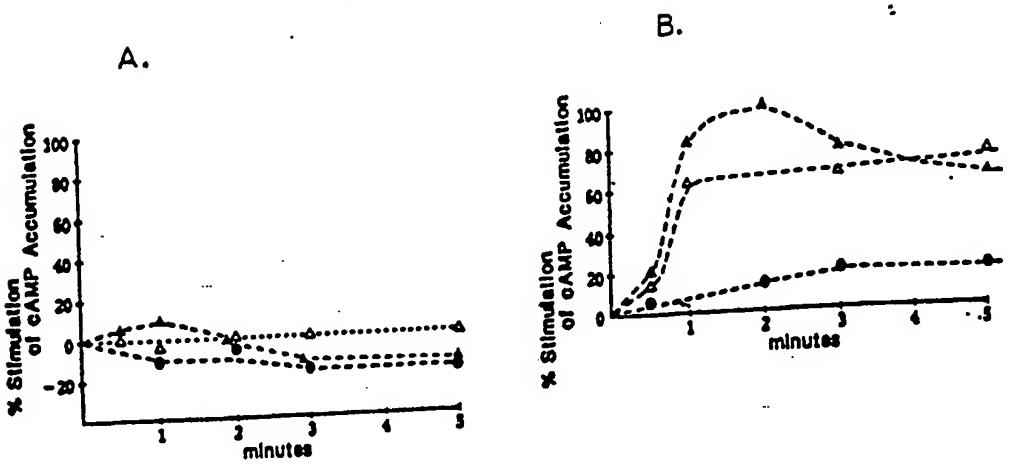

A.
B.
% Stimulation of cAMP Accumulation
minutes
100
80
60
40
20
0
-20
1
2
3
4
5

FIGURE 4A

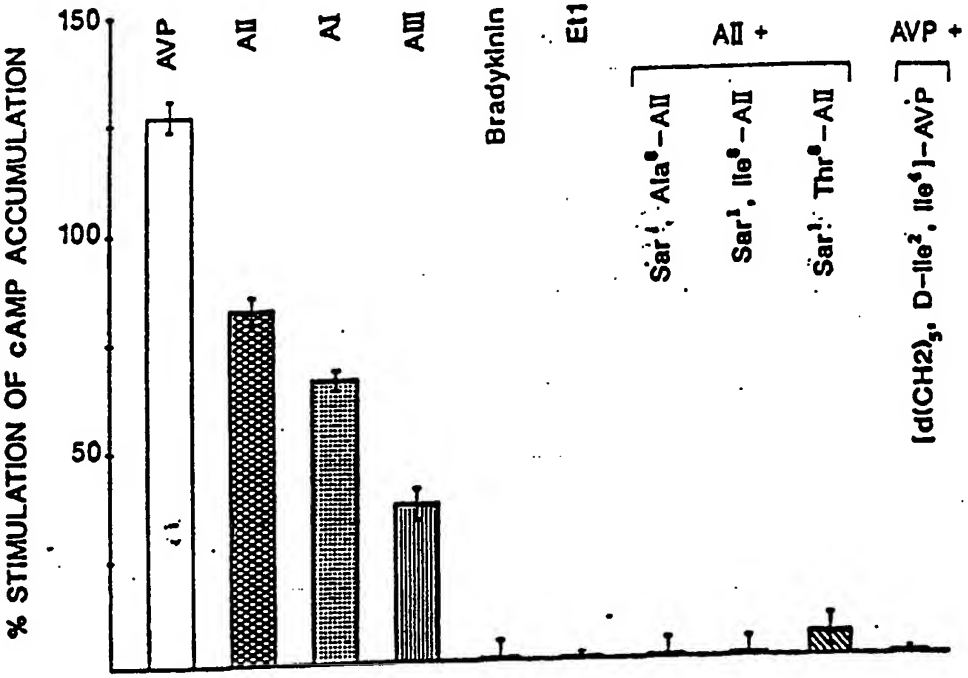

% STIMULATION OF cAMP ACCUMULATION
150
100
50
AVP
AII
AI
AIII
Bradykinin
Et1
AII +
AVP +
Sar¹, Ala⁸-AII
Sar¹, Ile⁸-AII
Sar¹, Thr⁸-AII
[d(CH2)5, D-Ile², Ile⁴]-AVP

FIGURE 4B

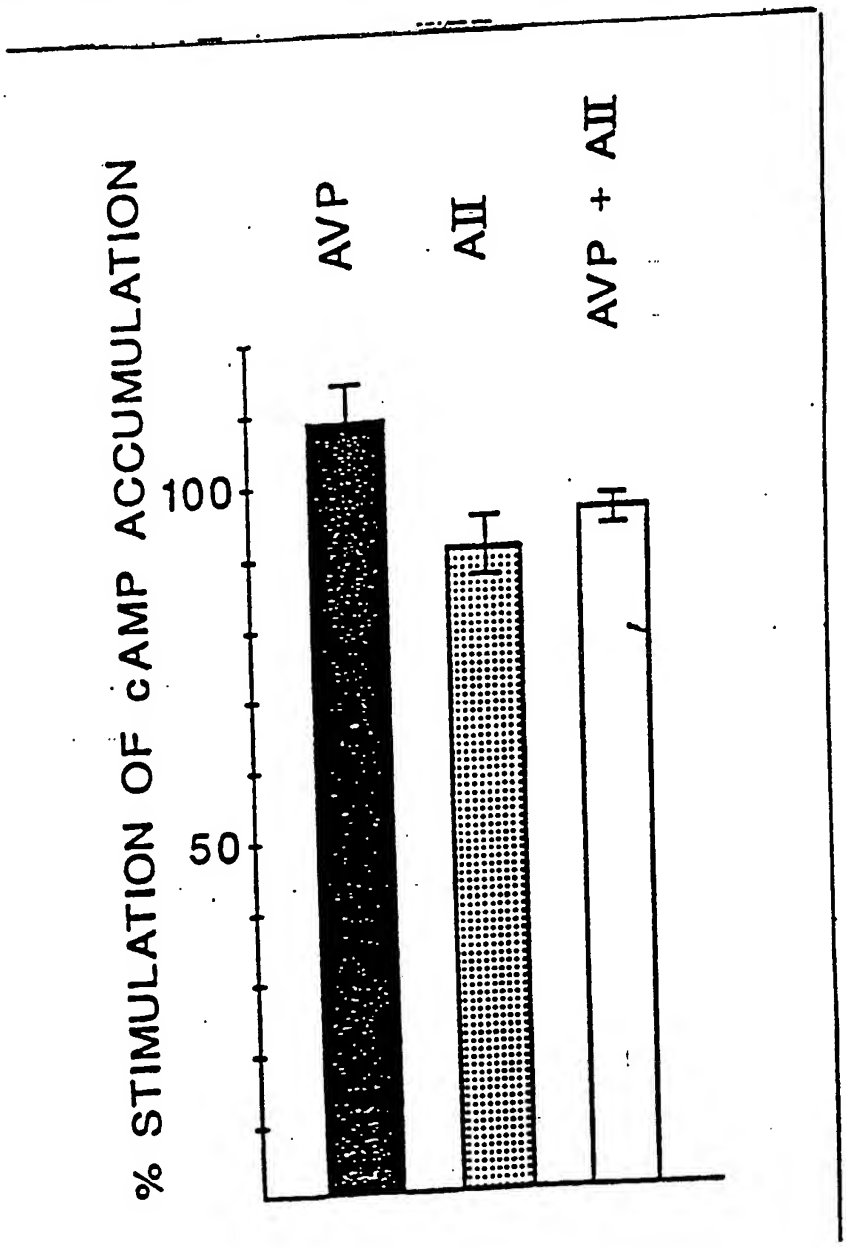


FIGURE 5A

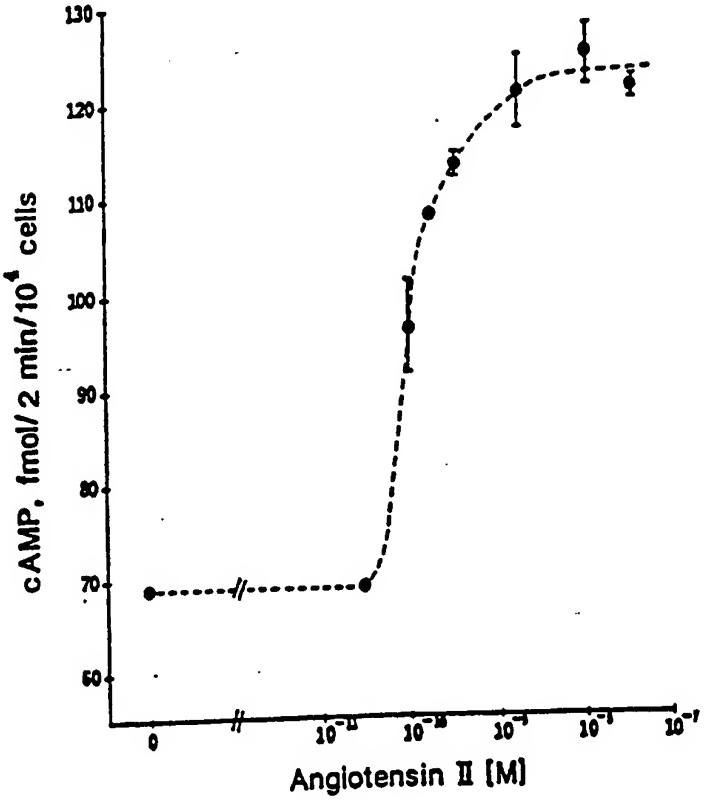

cAMP, fmol/ 2 min/10^4 cells
130
120
110
100
90
80
70
60
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
Angiotensin II [M]

FIGURE 5B

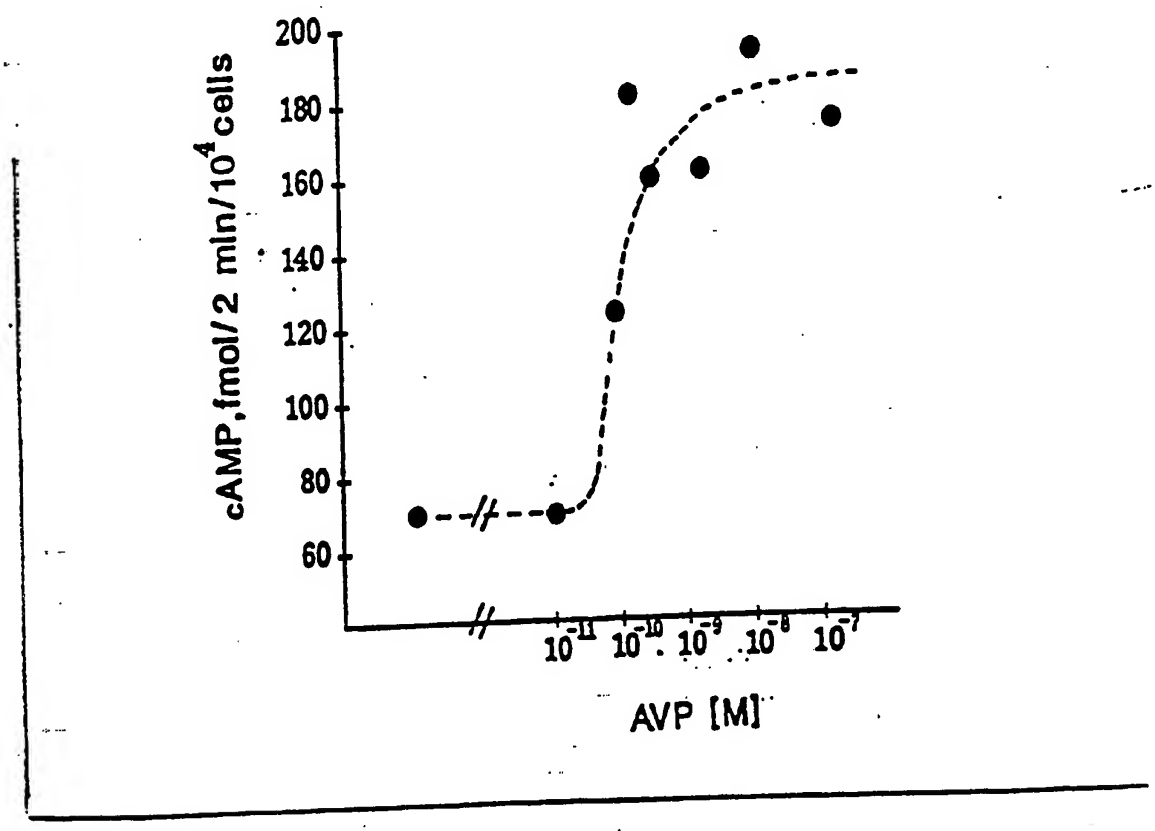

cAMP, fmol/2 min/10^4 cells
200
180
160
140
120
100
80
60
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
AVP [M]

FIGURE 6

Pharmacologic parameters of the AII/AVP receptor.

AGONISTS Compound	K_L(nM)	B_{max}	K_H(nM)	B_{max}	EC_{50}(nM)
AII	6.4	6.7	0.05	0.38	0.1
AVP	*5.9 +8.7	6.0	ND	ND	0.1
V2-type	109.0				

ANTAGONISTS Compound	K_i(nM)
V1-type	≥ 13,000
V1/V2	≥ 10,000

FIGURE 7A

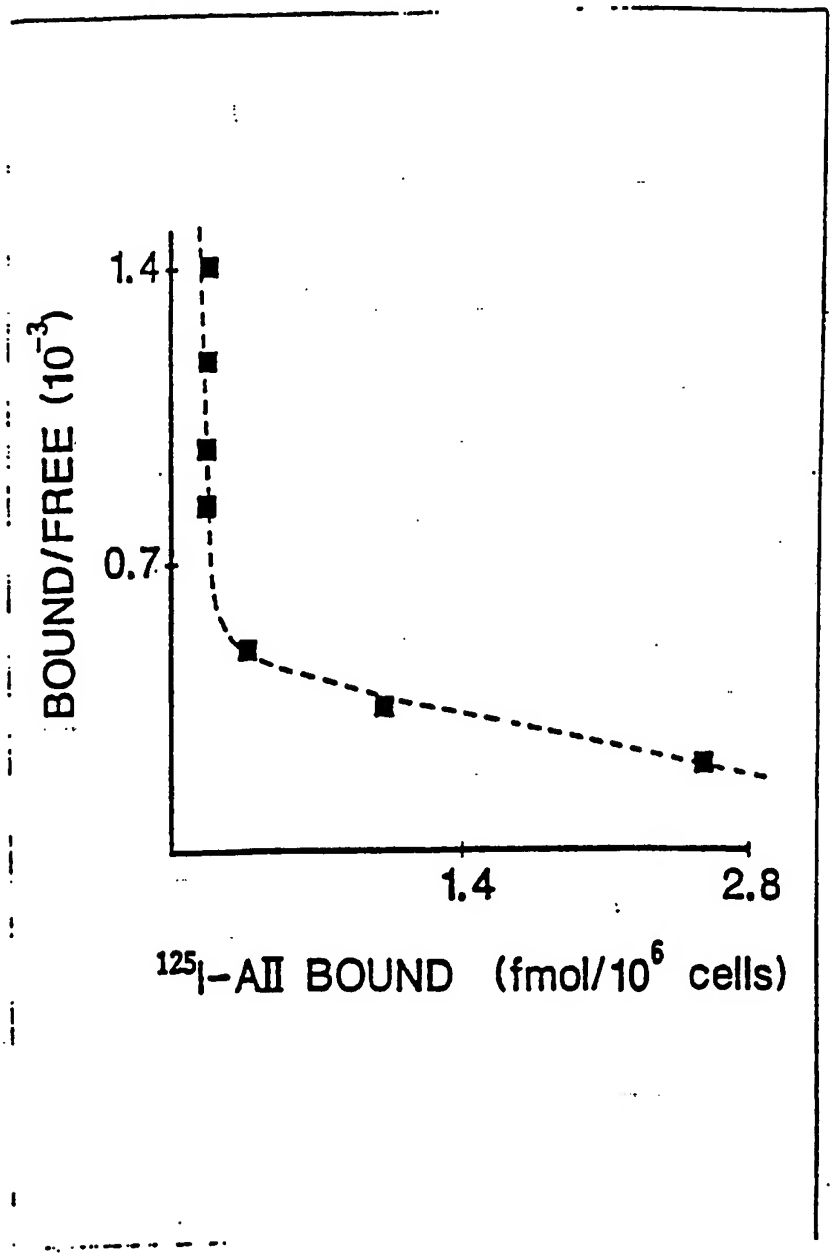


FIGURE 7B

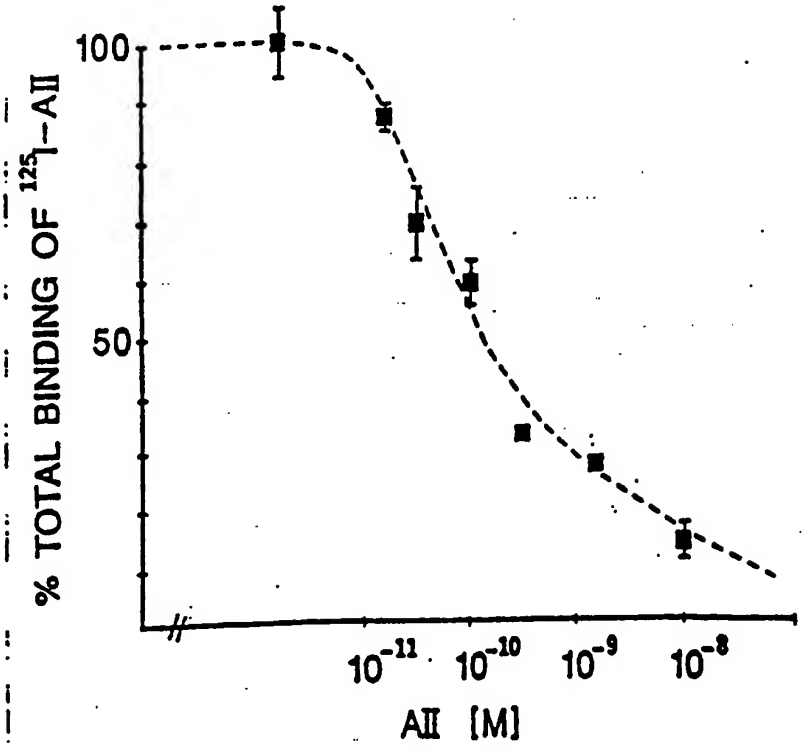


FIGURE 8A

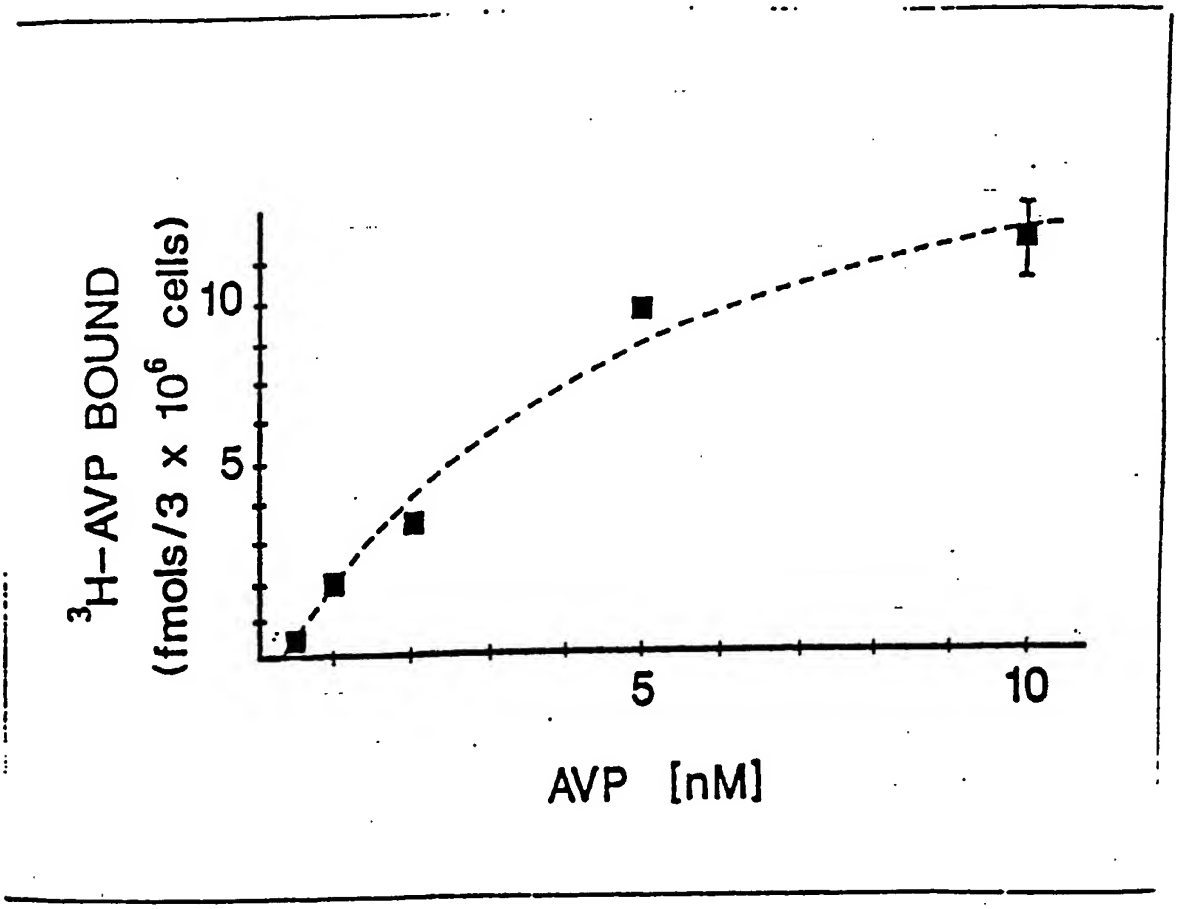


FIGURE 8B

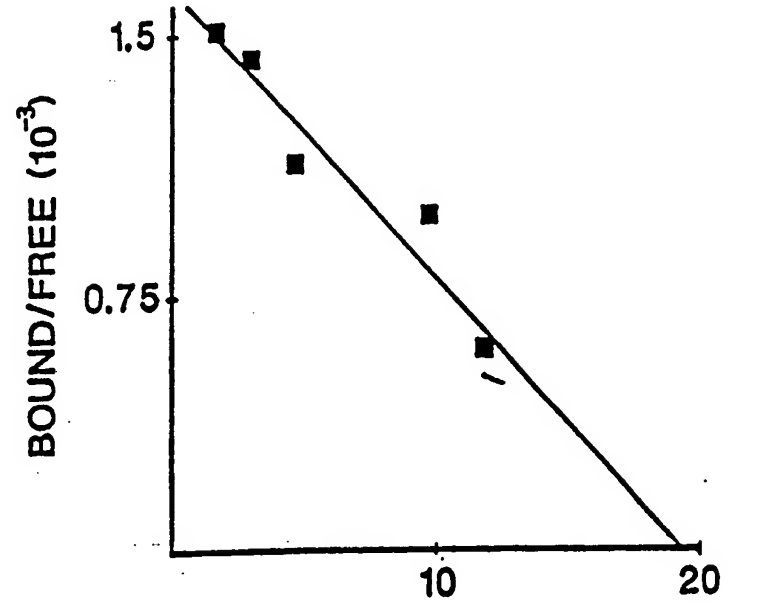


FIGURE 9

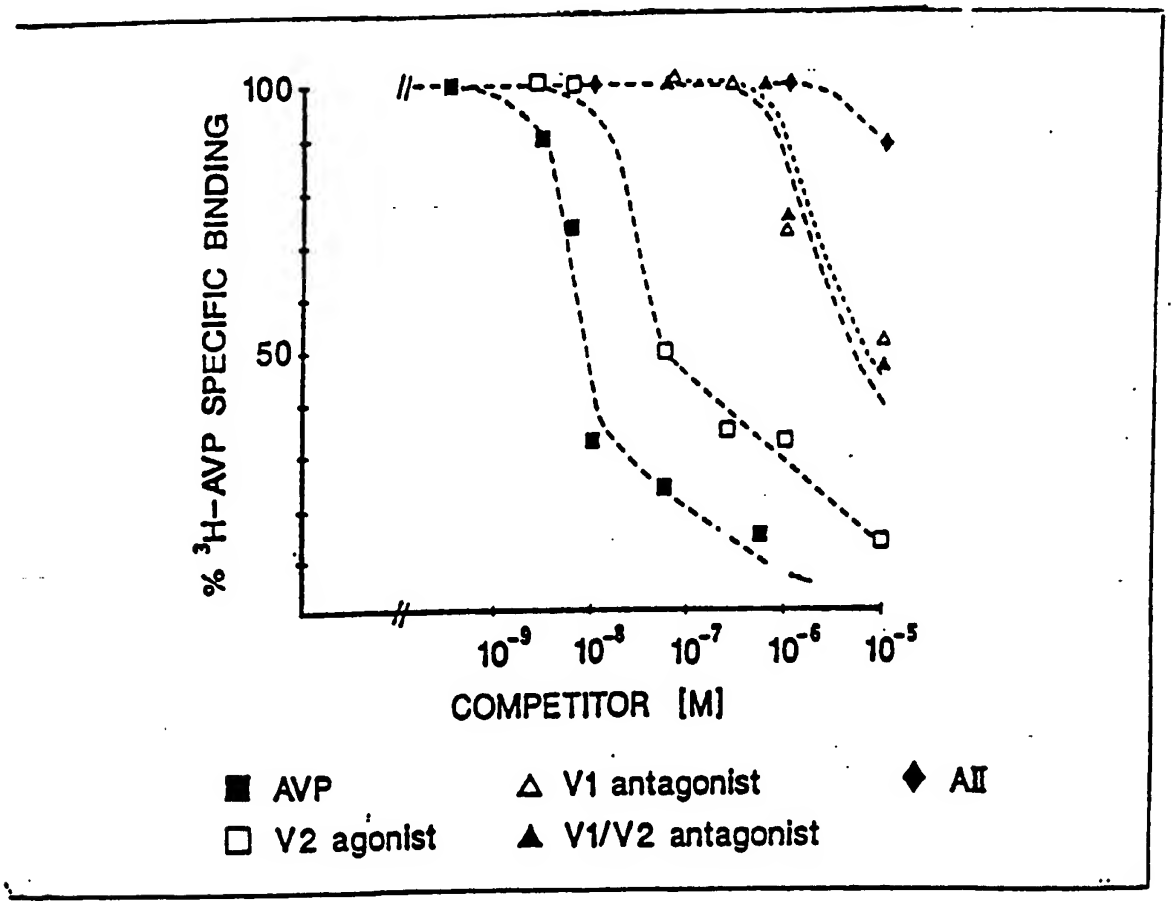

100
50
% ^{3}H-AVP SPECIFIC BINDING
10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5}
COMPETITOR [M]
AVP
V2 agonist
V1 antagonist
V1/V2 antagonist
AII

FIGURE 10

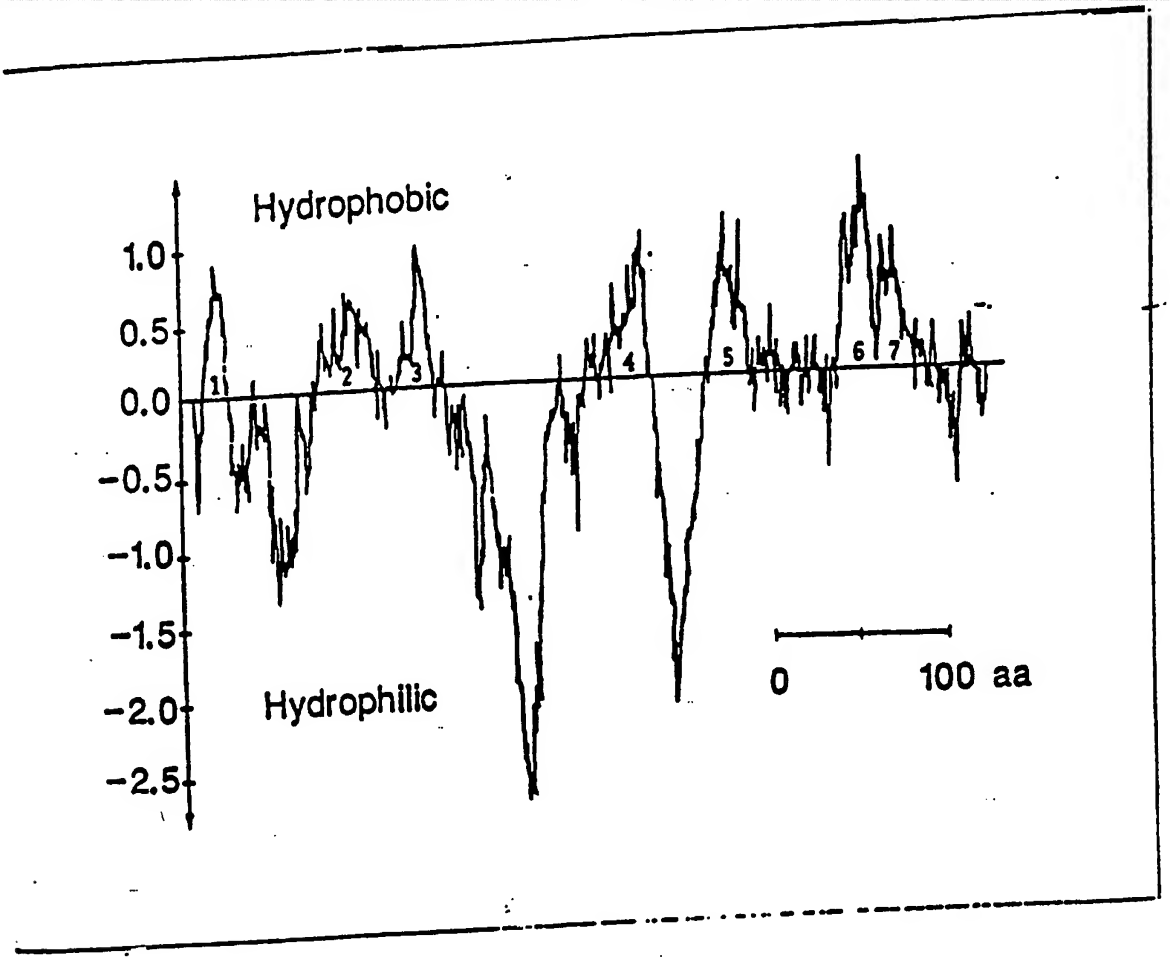

Hydrophobic
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
Hydrophilic
1
2
3
4
5
6
7
0
100 aa

FIGURE 11

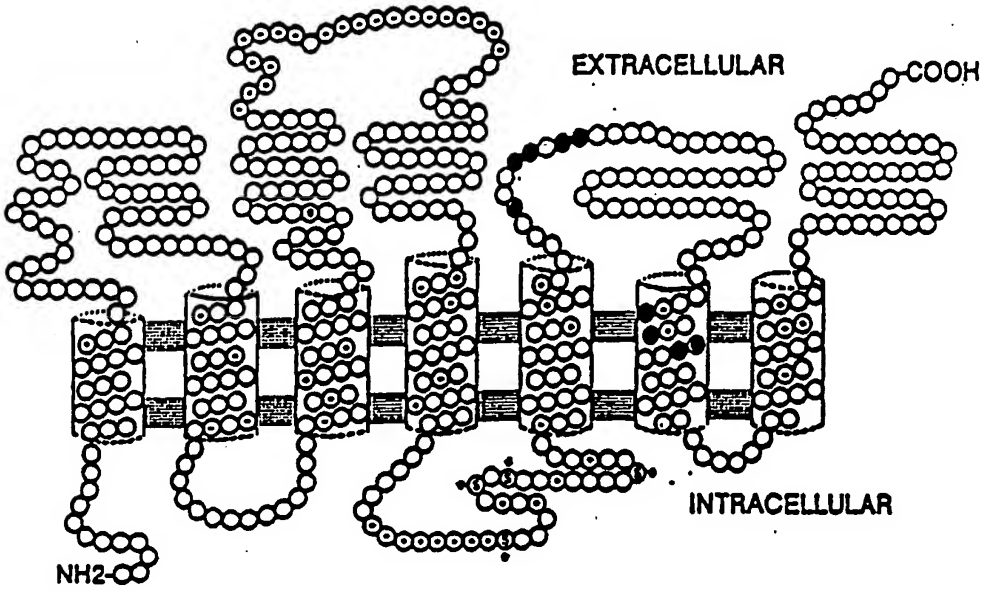
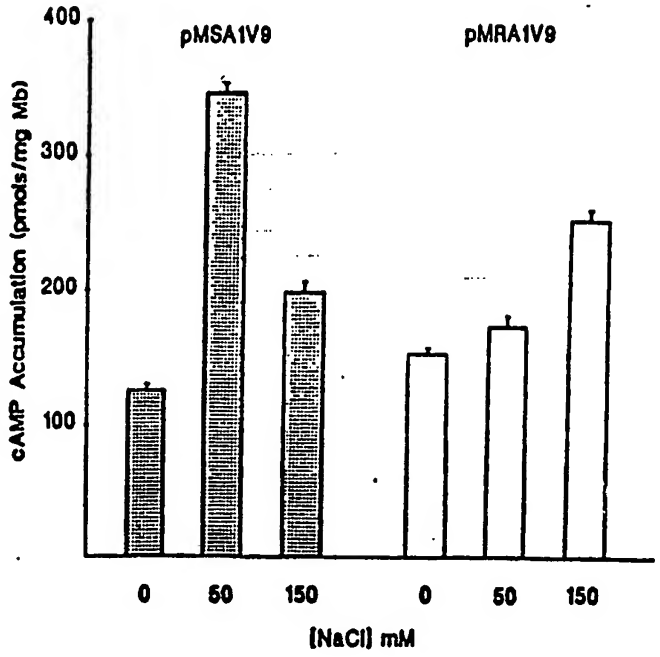


FIGURE 12

A


400
300
200
100
pMSA1V9
pMRA1V9
cAMP Accumulation (pmols/mg Mb)
0
50
150
0
50
150
[NaCl] mM

B

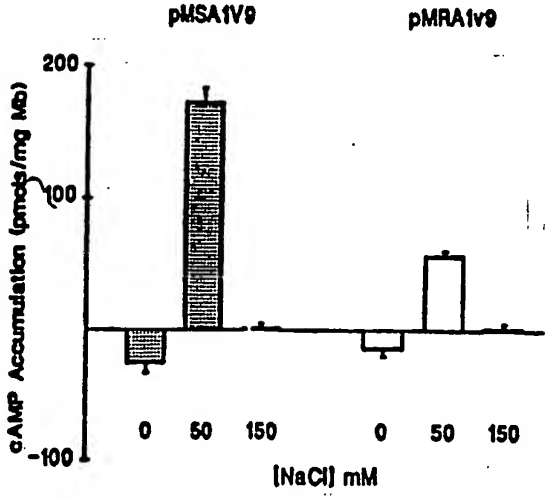

pMSA1V9
pMRA1v9
200
100
-100
cAMP Accumulation (pmols/mg Mb)
0
50
150
0
50
150
[NaCl] mM

FIGURE 13

Membrane	Hormone 0.1 μM	NaCl (mM)	cAMP Accumulation (pmols/mg Mb)	% Activity with respect to DR
DS	AVP	50	341±12	201 *
DR	AVP	50	170±6	100 *
DS	AII	50	170±4	298 *
DR	AII	50	57±1	100 *

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07786

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :530/350, 387.9; 435/7.8, 70.1, 240.1, 320.1; 514/2; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 387.9; 435/7.8, 70.1, 240.1, 320.1; 514/2; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	NATURE, VOL.357, ISSUED 28 MAY 1992, LOLAIT ET AL., "CLONING AND CHARACTERIZATION OF A VASOPRESSIN V2 RECEPTOR AND POSSIBLE LINK TO NEPHROGENIC DIABETES INSIPIDUS", PP. 336-339.	1-35
A	NATURE, VOL.351, ISSUED 16 MAY 1991, MURPHY ET AL., "ISOLATION OF A CDNA ENCODING THE VASCULAR TYPE-1 ANGIOTENSIN II RECEPTOR", PP. 233-236.	1-35

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
04 November 1992

Date of mailing of the international search report
02 DEC 1992

Name and mailing address of the ISA/
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07786

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C07K 13/00; C12P 21/02; G01N 33/53; A61K 37/02; C07H 21/04; C12N 15/00

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-35, drawn to angiotensin II/vasopressin receptor polypeptides, Class 530, subclass 350, antibodies against said polypeptides, Class 530, subclass 387.9, methods of making said polypeptides, Class 435, subclass 70.1, methods of using said polypeptides to test compounds, Class 435, subclass 7.8, therapeutic compositions comprising said polypeptides, Class 514, subclass 2, nucleic acids encoding said polypeptides, Class 536, subclass 27, and vectors and cells comprising said nucleic acids, Class 435, subclasses 320.1 and 240.1.
II. Claims 36-38, drawn to methods of detecting DNA associated with hypertension by identifying mutations in said DNA, Class 435, subclass 6.
III. Claim 39, drawn to methods of detecting DNA associated with hypertension by assaying cultured cells which express said DNA, Class 435, subclass 6.

The claims of groups I, II, and III are drawn to distinct methods and have a separate status in the art because of their recognized divergent subject matter. The first method relates to testing compounds for inhibitory activity against the angiotensin II/vasopressin V2 receptor. The second method relates to identifying DNA associated with hypertension by identifying mutations in the sequence of said DNA. The third method relates to identifying DNA associated with hypertension by assaying cells which express said DNA. PCT rules 13.1, 13.2, and 13.3 do not provide for multiple distinct methods within a single general inventive concept.